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Forschungseinheit Neurobiologie der Multiplen Sklerose

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Morphologische Analyse der distalen Auswirkungen des axonalen Transportdefizits in einem Tiermodell der Multiplen Sklerose

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ACTH	adrenocorticotropic hormone
ADP	adenosine diphosphate
ALS	amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
APP	amyloid precursor protein
ATP	adenosine triphosphate
BBB	blood brain barrier
Bcl-2	B-cell lymphoma 2
BDNF	brain derived neurotrophic factor
Ca^{2+}	Calcium
CASK	calmodulin-associated serin/threonine kinase
CFP	cyan fluorescent protein
CFP+	CFP-positive
CIS	clinically isolated syndrome
CNPase	cyclic nucleotide phosphodiesterase
CNS	central nervous system
COX	cytochrome oxidase
CSF	cerebrospinal fluid
DHODH	dihydroorotate dehydrogenase
DIS	dissemination in space
DIT	dissemination in time
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
Drp1	dynamin-related protein 1
EAAT1	excitatory amino acid transporter 1
EAE	experimental autoimmune encephalomyeliitis

EBNA	Ebstein-Barr Virus nuclear antigen
EBV	Ebstein Barr Virus
EDSS	expanded disability status scale
FAD	focal axonal degeneration
FAD+	Flavin adenine dinucleotide
FGF-2	fibroblast growth factor
Fis1	Fission protein 1
GA	glatirameracetate
Gd-	gadolinium nonenhancing
Gd+	gadolinium enhancing
GDAP1	gangliosid-induced differentiation associated protein
GED	GTPase effector domain
GLAST	glutamate aspartate transporter
GSK3b	AKT-gycogen synthase kinase 3b
GTP	guanine triphosphate
H+	hydrogen
H_2O_2	hydrogen peroxide
HLA	human leukocyte antigen
HO•	hydroxyl radicals
IFN	interferon
Ig	immunoglobin
IHC	immunohistochemistry
IL	interleukin
KIF	kinesin-1-family
KX	ketamine xylazine
LPS	lipopolysaccharide
MAC	mitochondria associated adherens complex
MAG	myelin associated glycoprotein
MBP	myelin basic protein
Mcl-1	Myeloid cell leukemia 1
Mff	mitochondrial fission factor
MFN	mitofusin
MHC	major histocompatibility complex
MiD	mitochondrial dynamics proteins
MIEF	mitochondrial elongation factor
Miro	mitochondrial Rho GTPase
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	Multiple Sclerosis
mtDNA	mitochondrial DNA
mtHSP70	mitochondrial heat shock protein 70
NaCl	sodium chloride
NAD	nicotine adenine
NAGM	Normal appearing gray matter

NAWM	Normal-appearing white matter
NMDA	*N*-Methyl-D-Aspartate
NGF	nerve growth factor
NGF	nerve growth factor
NO	nitric oxide
O2	oxygen
OCB	oligoclonal bands
OCP	oligodendrocyte precursor cell
OGT	O-GlcNAc Transferase
OPA	optic atrophy protein
OPN	osteopontin
pBAD	Bc-2-associated death promoter
PBS	phosphate buffered saline
PFA	paraformaldehyde
PI3K	phosphoinositide 3 kinase
PINK1	Pten-induced kinase 1
PLP	proteolipid protein
PML	progressive multifocal leukoencephalopathy
PNS	peripheral nervous system
PPMS	primary progressive multiple sclerosis
PSC	postsynaptic current
PSD95	post-synaptic density protein
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RRMS	relapsing remitting multiple sclerosis
S1P	sphingosine-1-phosphate
sEPSC	spontaneous excitatory postsynaptic currents
SPF	specific pathogen-free
SPMS	secondary progressive multiple sclerosis
Syn+	synapsin I-positive
TNF	tumor necrosis factor
TNFR1	tumor necrosis factor receptor 1
TRAK	trafficking kinesis proteins
UCP2	uncoupling protein 2
VCAMs	vascular cell adhesion molecules
VLA	very late antigen

Zusammenfassung

Multiple Sklerose (MS) ist eine der häufigsten neuroimmunologischen Erkrankungen, die bereits im jungen Erwachsenenalter zu bleibender Behinderung führen kann. Die Erkrankung ist gekennzeichnet durch entzündliche Läsionen, De- und Remyelinisierung sowie axonale Degeneration. Aktuelle Therapieoptionen wirken primär immunmodulatorisch oder immunsuppressiv. In der progredienten Phase, wenn die Entzündung größtenteils abgeklungen und axonaler Schaden und Degeneration die vorherrschende Pathologie ist, sind therapeutische Möglichkeiten rar. Demyelinisierung, Inflammation und mitochondriale Dysfunktion führen zu oxidativem Schaden und metabolischem Ungleichgewicht in den Axonen. Im Tiermodell Experimentelle Autoimmunenzephalomyelitis (EAE) konnte gezeigt werden, dass Transportdefizite, die mit der Entzündung einhergehen, weitverbreitet und anhaltend sind. Während in der akuten Phase der Transport in beide Richtungen unterbrochen ist, bleibt in der chronischen Phase der anterograde Transport stärker beeinträchtigt als der retrograde.

In der vorliegenden Arbeit habe ich mich mit den Auswirkungen des chronischen Transportdefizits auf die mitochondriale Verteilung in Axonkollateralen und Boutons/Synapsen beschäftigt. EAE wurde in transgenen Tieren, welche an Mitochondrien gebundenes fluoreszierendes Protein exprimieren, induziert. Mittels Spinalganglieninjektion eines viralen Vektors konnte eine definierte Population von Axonen, die eine entzündliche Läsion durchqueren und in der lumbalen grauen Substanz Kollateralen abgeben, markiert werden. Synapsen und Entzündungszellen wurden durch Immunfluorezenzfärbung visualisiert.

In akuter EAE hatten kurz anhaltende Transportunterbrechungen keinen Einfluss auf den distalen Gehalt an Mitochondrien, während langanhaltende Transportdefizite als Resultat chronischer Neuroinflammation zu einer Reduktion der distalen Mitochondrienzahl und damit zu einer energetischen Minderversorgung des distalen Nervenastes führten. Darüberhinaus zeigten Axone ohne Mitochondriendepletion ein anderes Verteilungsmuster als die Kontrollen mit einem Shift der Mitochondrien aus den Boutons in die dazwischenliegenden Abschnitte. Die Anzahl und das Volumen der Mitochondrien innerhalb der Boutons waren reduziert, wohingegen die Anzahl der Boutons unverändert blieb. Diese Ergebnisse deuten daraufhin,

dass die Transportdefizite zu einer distalen mitochondrialen Depletion und damit Affektion der grauen Substanz beitragen, die bei EAE und MS beobachtet werden kann. Je mehr klinische Defizite sich in chronisch progredienter MS über die Zeit anhäufen, desto weniger sprechen sie auf antiinflammatorische Medikation an. Zukünftig müssen neue therapeutische Ansätze geschaffen werden, die zu einer Wiederherstellung des axonalen Transports, der axonalen Homöostase und der mitochondrialen Funktion beitragen können.

Summary

Multiple Sclerosis (MS) is one of the most common causes for neurological disability in young adults, hallmarked by inflammatory lesions, de- and remyelination and axonal degeneration. Current treatment options are primarily directed at immunomodulation or immunsuppression. In the progressive phase, when inflammation has abated and axonal injury and degeneration is the dominant pathology, treatment options are sparse. Demyelination, inflammation and mitochondrial dysfunction lead to oxidative damage and metabolic imbalance within the axons, which ultimately results in axonal degeneration. In the animal model experimental autoimmune encephalomyelitis (EAE) it could be shown that transport deficits linked to inflammation are pervasive and continuous. While acutely anterograde and retrograde transport are both affected, in chronic EAE mostly anterograde transport is reduced.

The implications of the chronic transport deficits on mitochondrial distribution in axon collaterals and synapses were subject of my thesis. We induced EAE in genetically modified mouse lines that express mitochondrially targeted cyan fluorescent protein. Via dorsal root ganglion injection of a viral vector for a fluorescent protein I was able to follow a selective set of axons through an inflammatory lesion passing off collaterals into the gray matter. Synapses and inflammatory cells were visualized by immunofluorescence.

In acute EAE, short lasting transport interruptions do not affect the distal mitochondrial content, while longer lasting transport deficits as observed in chronic neuroinflammation decrease the distal mitochondrial content and thereby diminish the energy supply of the distal axonal arbor. Moreover, healthy appearing axons not depleted of mitochondria showed a different mitochondrial distribution with depleted boutons and increased content in the extrasynaptic parts. While mitochondrial number and volume within the boutons was decreased, the number of boutons was unaltered. These results indicate that distal mitochondrial depletion might contribute to gray matter pathology concomitant with clinical deficits seen in EAE and MS. As clinical deficts accumulate over time and are unresponsive to

anti-inflammatory treatment in progressive MS, new therapeutic approaches that can recover axonal transport, restore the axonal homeostasis and supporting mitochondrial function should be explored.

1 Introduction

1.1 Early History of MS

As Robert Carswell pondered over a spinal cord specimen of a deceased patient in 1838, he noted “a peculiar diseased state” (Carswell, 1838; T. J. Murray, 2009). He saw brown atrophied patches, which were firm to the touch. The only clinical fact he knew about the patient formerly bearing those scars was that he was paralyzed. Before proceeding with many other pathological curiosities on his table, he produced a drawing of the cord and included it on atrophy plate IV of his Pathological Anatomy: Illustrations of Elementary Forms of Disease. This is regarded as the first illustration of the pathology of Multiple Sclerosis (MS) (T. J. Murray, 2005). It took another 30 years until Jean-Martin Charcot gave this peculiar disease an apt name: la sclérose en plaque disseminée. The debatably earliest case described is that of Lidwina of Schiedam, born in 1380. After a fall while ice skating she developed a series of symptoms that are typical for the disease course of MS: difficulty walking, visual problems, headaches, and progression to a complete paraparesis, only to name a few (Maeder, 1979). Since back in 1380 she had no physician capable of diagnosing and treating her, she was canonized by the church and thought to suffer for the sins of others. Following this perhaps earliest case report of what is today called MS, many other cases appeared throughout history, which were only correctly diagnosed centuries later and include Auguste d’Esté and Heinrich Heine, just to mention two (A. Compston, 1998; Jellinek, 1990; T. J. Murray, 2005; Stenager, 1996).

In the mid-19th century, Jean-Martin Charcot and Alfred Vulpian made it their mission to characterize and organize patients they have encountered in Paris’ state-of-the-art neuropsychiatric hospital, the Salpêtrière, to establish a classification of neurological diseases by matching clinical and pathological descriptions (Kumar, Aslinia, Yale, & Mazza, 2011; T. Jock Murray, 2009). In the process of their work, they presented the first three official cases of MS, a disease that had occupied the attention of generations of physicians before them. Hallmark symptoms were tremor, slurred speech and abnormal eye movements, later termed Charcot’s Triad.

1.2 The cellular image of MS

Light microscopy became popular at the beginning of the 20th century, allowing scientists to take a closer look at tissues and to identify structures they never knew existed. Cajal and Golgi (1906) discovered a dye to selectively visualize nerve cells (Glickstein, 2006). While Rudolf Virchow first introduced the term myelin and described the sheaths around nerve fibers, the idea that these might be glial cells to insulate nerve cells was first suggested by Santiago Ramón y Cajals brother in the early 1900s (Cajal, 1913; A. Compston, 1998). Louis Ranvier characterized the myelin sheath and James Dawson in Edinburgh described its breakdown in MS and the proximity of the inflammation to blood vessels (Dawson, 1916; T. J. Murray, 2005).

The middle of the 20th century was a period, in which animal models for diseases came into focus. Thomas Rivers (1935) injected myelin into lab animals and thereby produced a number of symptoms that were similar to those present in MS (Rivers & Schwentker, 1935). This model was called experimental autoimmune encephalitis (EAE) and is still one of the most commonly used models for MS. In 1942, Elvin Kabat at Columbia University first used electrophoresis to show that the cerebrospinal fluid (CSF) of MS patients contained a higher proportion of gamma globulin (Kabat, Moore, & Landow, 1942). His study paved the way for the discovery of oligoclonal bands (OCBs), which were subsequently identified by Lowenthal, who thereby made the first contribution to non-clinical diagnostic criteria (Lowenthal, Vansande, & Karcher, 1960).

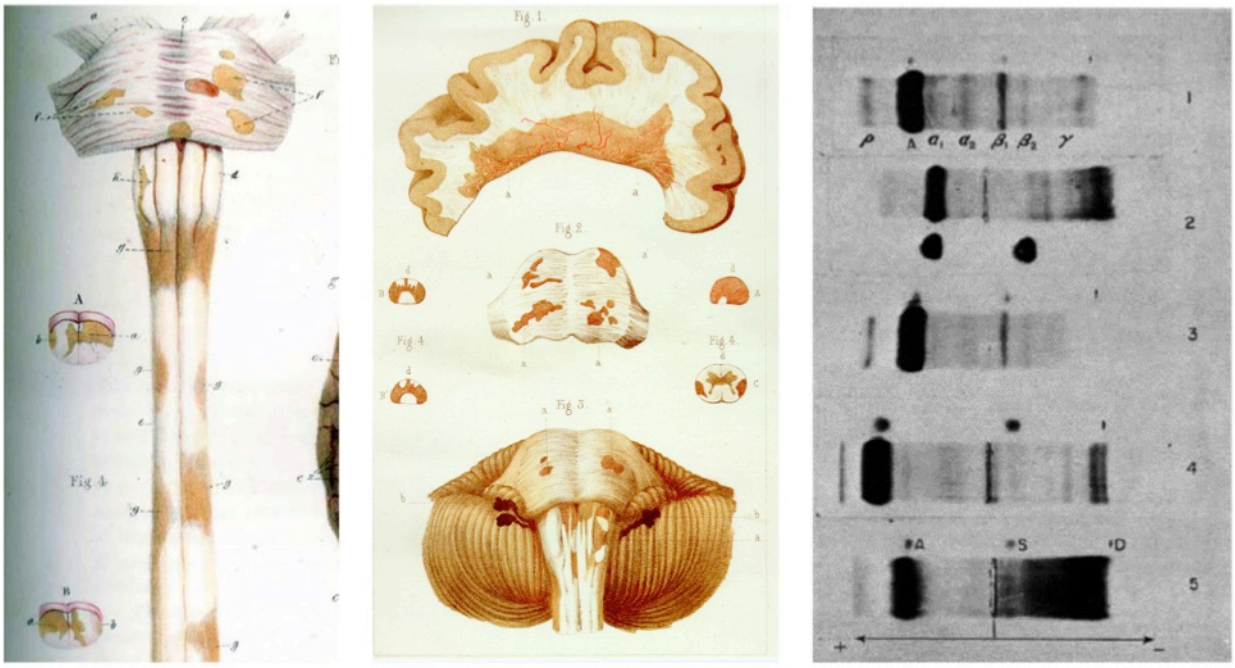


Fig. 1-1 Historical images of MS
(A) Carswell's illustration of "a peculiar disease state of the cord, and pons Variolii" affected with multiple sclerosis, included in his atrophy plate IV, brown patches visible in the pons and the spinal cord, a cross section showing the extent of the lesion from superficial to profound (Carswell, 1838) source: http://special.lib.gla.ac.uk/exhibns/month/oct2003.html, (B) Charcot's illustration of his cases of sclerose diseminée presented in his lectures at Salpêtrière, plate II, (Ordenstein, 1868) (C) gel micro-electrophoresis showing oligoclonal bands, 1 is from a patient with MS, 3 is normal (Lowenthal et al., 1960) rights were obtained from Wiley.

1.3 Etiology and Epidemiology

In the past plenty of theories on the etiology of MS have emerged and disappeared just as fast. Leyden proposed a vascular pathology in 1863, because plaques have often been found in proximity to small veins (Leyden, 1863). In the early 1900s Marburg suggested a toxin theory due to myelinolytic factors, whereas Steiner and colleagues assumed that spirochetes, small spiral bacteria, might be the cause of MS (Harding, Dobin, Pollock, & Ruge, 1959; Marburg, 1906; Schmidt, 2012). By the 1960s, there were two main theories how the disease originates. One proclaimed that MS is an autoimmune disease, in which leucocytes attack the myelin basic protein (MBP) and thus destroy the myelin sheath of the nerve cells. The other one proposed the concept of a viral cause, which was thought to act as a trigger for the immune system (T.

Jock Murray, 2009). Both concepts are still debated today and do not necessarily exclude each other.

1.3.1 Geographical factors

While the etiology of MS still remains largely obscure epidemiological studies have given a first insight. After World War II large amounts of data on MS in young soldiers revealed a marked geographical gradient of disease prevalence. In the northern parts of Europe and the United States the disease was more common than in areas around the equator (Acheson, Bachrach, & Wright, 1960; A. Compston, 1998). A revision of this study with a new cohort of veterans showed an attenuation of this latitude gradient (Alonso & Hernán, 2008). The estimated prevalence of MS in Germany depends on methodology, population and region of the study and varies between 102,000 to 143,000 patients, corresponding to an average prevalence rate of 149.1 per 100,000 inhabitants (Hein & Hopfenmüller, 2000; Höer et al., 2014; S. Poser & Bauersfeld, 1995). A recent analysis demonstrated an increase in prevalence of MS in Europe, which might be due to better-defined diagnostic criteria and improved detection methods (Dippel, Mäurer, Schinzel, Müller-Bohn, & Larisch, 2015). In how far this is also determined by a true rise in prevalence remains unclear. Factors such as an infection with the Ebstein-Barr Virus (EBV), exposure to sun and Vitamin D levels and genetics could contribute to the geographical gradient.

1.3.2 Gender and MS

MS most often occurs in the age group between 20 and 40 (Flachenecker et al., 2008). The incidence in women is approximately twice as high as in men (Noseworthy, Lucchinetti, Rodriguez, & Weinshenker, 2000). Newer studies show the female-to-male ratio increasing from 1.5 to 2.5, between the 1960s and the 2000s (Alonso & Hernán, 2008). It is well known that hormonal variations induced by pregnancy and the post-partum period can lead to alterations in the relapse rate. Moreover there is accumulating evidence suggesting that in-vitro

fertilization could constitute a pathogenetic factor in MS (Hellwig et al., 2008; Ladwig, Dunkl, Richter, & Schroeter, 2016).

1.3.3 Genetics in MS

The cumulated occurrence of MS in families and concordance rates in monozygotic twins of 25.3% and in dizygotic of 5.4% lead to the assumption that a certain predisposition for the disease can be inherited (Eichhorst, 1896; Schapira, Poskanzer, & Miller, 1963; Willer et al., 2003). Through epidemiological studies and gene analyses, over 100 genetic variants and transmission patterns have been discovered (Hemmer, Kerschensteiner, & Korn, 2015). One group found an association with the segregation of the human leukocyte antigen (HLA)-DRB1*15 allele (Ebers, Sadovnick, & Risch, 1995; Ghabanbasani et al., 1995). A multicenter study revealed an increased risk for developing MS in people expressing HLA class II alleles DRB*1501, DRB1*0301, and DRB*1303 on their innate immune cells and a decreased risk for HLA class I allele A2 (Sawcer et al., 2011). Kalman and Lublin already concluded in 1999, which is still the prevailing assumption, that MS has a complex pattern of inheritance that is not determined by a single gene but rather by multiple alterations in genes responsible for immune response (Kalman & Lublin, 1999).

1.3.4 The Vitamin D theory

The north-south gradient of disease frequency has been suggested to be due to higher exposure to sunlight and therefore elevated Vitamin D levels (VanAmerongen, Dijkstra, Lips, & Polman, 2004). Indeed, studies have revealed that high Vitamin D levels are associated with a lower risk for MS (Kassandra L. Munger, Levin, Hollis, Howard, & Ascherio, 2006). More recently low levels of Vitamin D have been shown to be associated with a higher relapse rate (Runia, Hop, de Rijke, Buljevac, & Hintzen, 2012). Vitamin D has also proven beneficial in EAE. Especially it is said to have anti-inflammatory properties and lead to a decreased ratio of T1/T2 interleukins (Cantorna, Hayes, & DeLuca, 1996; Soleimani, Jameie, Mehdizadeh, et al., 2014).

These effects might partly be mediated by natural killer T (NKT)-cells via interleukin-4 (IL-4) secretion (Waddell, Zhao, & Cantorna, 2015).

1.3.5 Infection and MS

Pierre Marie introduced the idea of an infectious cause for MS in 1884 and to this day it has neither been clearly proven nor disproven (A. Compston, 1998). However there is evidence for at least a partial role of viruses. HHV-6 infiltrates the CNS in early childhood, persists livelong and is known to have a cross-reactivity with the MBP (Broccolo, Fusetti, & Ceccherini-Nelli, 2013; Tejada-Simon, Zang, Hong, Rivera, & Zhang, 2003). EBV seems to play a role as well. In a study conducted by Sundström and colleagues, a high antibody activity against Ebstein-Barr Virus nuclear antigen (EBNA)-1 was associated with an elevated risk for MS, whereas a high titer against the viral capsid antigen VCA was linked to a lower risk (Sundström et al., 2004). In addition, the time of infection seems to play a role (Pender & Burrows, 2014).

1.3.6 Nutrition and MS

Wekerle and colleagues suggested the initiation of MS pathology might be influenced by the gut microbiome (Berer, Mues, et al., 2011). Using a mouse model that develops spontaneous EAE they found that a line of mice housed in a germ free environment were protected from EAE throughout their lives compared with animals held under specific pathogen-free (SPF) environment. The crucial difference was that the germ free mice could not develop a gut flora. When the gut microbiome was transferred from the SPF- to the germ free mice they developed spontaneous EAE just the same. A cross reaction between bacterial species in the intestinal flora could prime an autoimmune reaction by supporting the differentiation of interleukin 17 (IL-17) secreting T-lymphocytes (Berer, Mues, et al., 2011). A Japanese group around Yamamura has suggested such a link already in 2008, when they treated a group of mice with non-absorbing antibiotics starting one week prior to immunization with EAE and found an amelioration of the clinical course of EAE, a reduction of mesenteric Th17 cells and a lower pro-inflammatory cytokine secretion from the draining lymph nodes (Yokote et al., 2008).

Furthermore, ghrelin, a gastric “hunger” hormone has been shown to influence the clinical severity of EAE (Theil et al., 2009).

Moreover a possible link between dietary sodium intake and increased disease activity in EAE and MS has been a recent focus of research. Macrophage infiltration of the CNS and pro-inflammatory phenotype polarization were shown to be elevated with a diet high in sodium chloride (Hucke et al., 2016). Binger, Kleinewiedfeld and colleagues showed that salt increases Th17 cells and LPS-induced macrophages and reduces anti-inflammatory macrophage activation (Binger et al., 2015; Kleinewietfeld et al., 2013). In an observational study Farez and colleagues were able to demonstrate a link between high sodium intake and clinical and radiological disease activity in human MS (Farez, Fiol, Gaitán, Quintana, & Correale, 2015).

In summary, MS seems to have a multifactorial etiology in which a genetic predisposition and environmental factors contribute to triggering an autoimmune response against the myelin sheaths.

1.4 Presentation and Course of disease

The course of disease and the extent to which the symptoms affect the patient are individually variable. Inflammatory MS lesions can affect the entire central nervous system (CNS) and thus present with a wide variety of symptoms, depending on the location of the lesions. Nevertheless there are predominant areas that are more often affected than others and symptoms that are more likely to appear early on in the course of disease, such as optic neuritis, pareses and sensory disturbances and those that occur later, such as pain syndromes, spasticity, ataxia, loss of abdominal reflexes, optic nerve atrophy and bladder or bowel dysfunction (C. M. Poser, 1965, 1980). Impaired sexual function, fatigue, depression and cognitive dysfunction can also present early on and be very debilitating.

Disease progression can be just as variable as the symptoms. Patients can be mildly affected or end up paralyzed. We can differentiate between at least three major forms: 1) a relapsing-

remitting disease (RRMS), which is marked by intermittent exacerbations, with or without residual symptoms, 2) a secondary chronic progressive form (SPMS) which develops from RRMS in 85% of the cases after 15 years and is characterized by a steady, often slow disease progression with or without superimposed exacerbations and/or plateaus, and 3) a primary chronic progressive form (PPMS) hallmarked by a progressive worsening without a preceding phase of exacerbations (Lublin & Reingold, 1996).

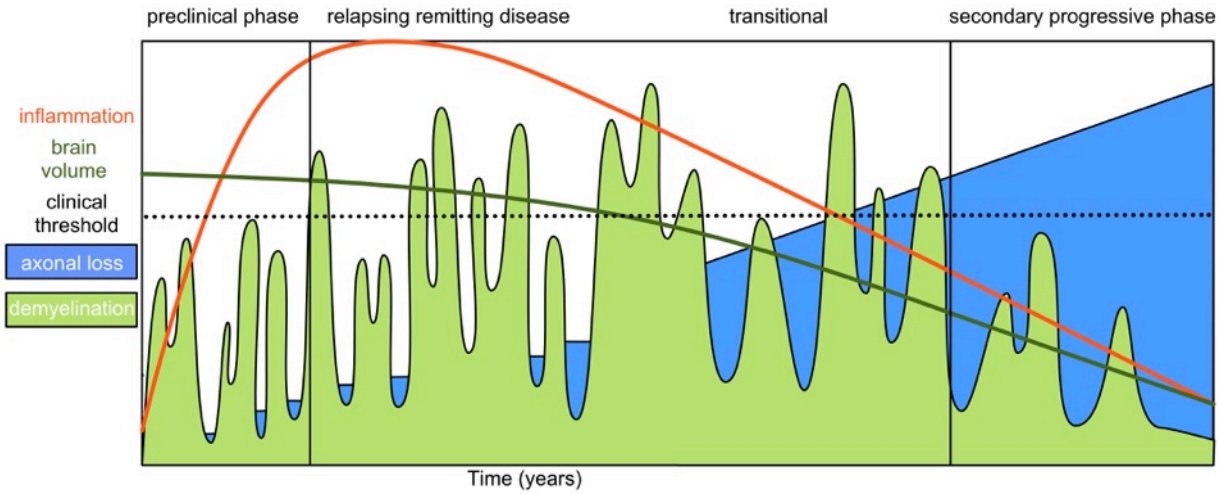


Fig. 1-2 Disease progression
Multiphasic disease course of MS displaying characteristics of different phases, with demyelination displayed in green, axonal loss in blue, clinical threshold (dotted line), brain volume (dark green line), inflammation (red line). During the preclinical phase: high inflammation, not yet clinically apparent, lesions on MRI can occur, axon loss begins. During relapsing-remitting disease, clinical exacerbations alternate with phases of remission, while inflammation peaks and then declines and brain volume loss begins. RRMS slowly transitions into secondary progressive disease. Exacerbations are superimposed on a continuous clinical progression until relapses stop, inflammation wanes, brain volume loss and axonal loss are extensive. Adapted from (Alastair Compston & Coles, 2002; Hans Lassmann & van Horssen, 2011).

1.5 Diagnostic criteria

Even 150 years after the first mention of MS, diagnosis is often difficult to make right away. The diagnosis is based on clinical and paraclinical assessments. Magnetic resonance imaging (MRI), neurophysiological testing, CSF and blood analysis have proven to be most relevant. Dissemination in time (DIT) and space (DIS) has to be demonstrated and other likely diagnoses have to be ruled out. The McDonald diagnostic criteria serve as guidelines (McDonald et al.,

2001; Polman et al., 2011). A single clinical event, that is likely to be caused by a demyelinating process without dissemination in time is called clinically isolated syndrome (CIS), which in 85% of the cases marks the initial appearance of MS (D. Miller, Barkhof, Montalban, Thompson, & Filippi, 2005).

Clinical (attacks)	**Lesions**	**Additional Criteria to make Diagnosis**
2 or more 	Objective clinical evidence of ≥ 2 lesions of 1 lesion with reasonable historical evidence of a prior attack	None, clinical evidence alone will suffice; additional evidence desirable but must be consistent with MS
2 or more 	Objective clinical evidence of 1 lesion	DIS, demonstrated by: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord); or await a further clinical attack implicating a different CNS site
1 	Objective clinical evidence of ≥ 2 lesions	DIT, demonstrated by: Simultaneous presence of asymptomatic gadolinium - enhancing (Gd+) and nonenhancing (Gd-) lesions at any time; or a new T2 and/or Gd+ lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or await a second clinical attack
1 (CIS) 	Objective clinical evidence of 1 lesion	DIT and DIS, demonstrated by: For DIS: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord); or await a second clinical attack implicating a different CNS site; For DIT: Simultaneous presence of asymptomatic Gd+ and Gd- lesions at any time; or a new T2 and/or Gd+ lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or await a second clinical attack
0 	Insidious neurological progression suggestive of MS (PPMS)	One year of disease progression plus 2 of 3 of the following criteria: 1. Evidence for DIS in the brain based on ≥ 1 T2 lesions in the MS-characteristic (periventricular, juxtacortical, or infratentorial) regions 2. Evidence for DIS in the spinal cord based on ≥ 2 T2 lesions in the cord 3. Positive CSF (evidence of OCBs and/or elevated IgG index)

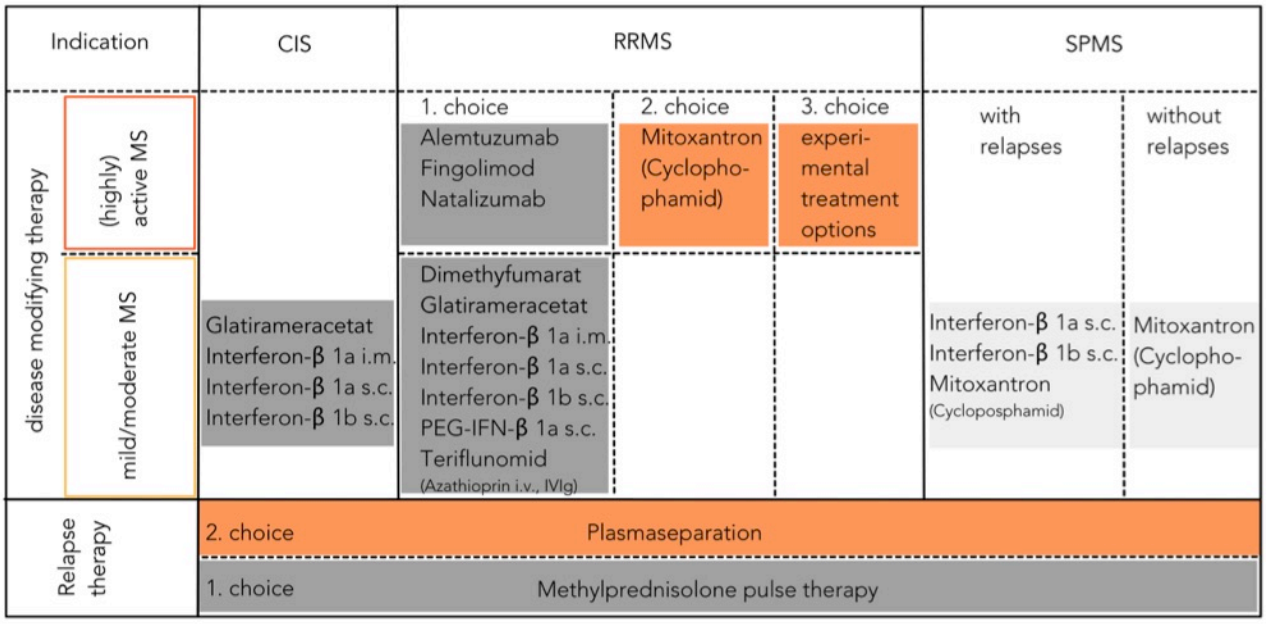
Tab. 1-1 McDonald diagnostic criteria
Adapted from (Polman et al., 2011), rights obtained from John Wiley and Sons.

1.6 Treatment

1.6.1 Treatment of acute exacerbation

For almost a century empirical therapy was the basis for treatment. From arsenic, mercury, auric chloride, zinc sulfate to belladonna and ergotamines, every available remedy was tried (Schmidt, 2012). The relapsing remitting nature of the disease made it difficult to distinguish between real improvement and normal disease development. In 1961 with the universalized introduction of controlled studies, injecting adrenocorticotropic hormone (ACTH) proved superior to a saline-treated group and finally provided proof of an effective medication (Berkovich, 2013; H. Miller, Newell, & Ridley, 1961).

Later on ACTH was replaced by cortisone, which is still the gold standard medication for an acute exacerbation. Glucocorticoids act anti-inflammatory, anti-edematous and immunosuppressive. The blood-brain-barrier (BBB) disruption quickly abates and the effect lasts approximately six to nine weeks. It can serve to reduce relapse duration, but cannot alter the disease course or residual disability (Hoffmann, 2012; Ontaneda & Rae-Grant, 2009). Additional treatment options in the acute phase are plasma exchange and immunoadsorption, especially in steroid non-responsive cases (Koziolek et al., 2012; Ontaneda & Rae-Grant, 2009).



Tab. 1-2 Therapeutic guidelines by the DMSG
Adapted from Leitlinien für Diagnostik und Therapie in der Neurologie: Diagnose und Therapie der Multiplen Sklerose, effective as of January 2012, valid until 2017, translated into English

1.6.2 Disease modifying therapy in mild/moderate RRMS

First described as antiviral substances by Isaacs and Lindenmann, interferons (IFNs) have been the first substance to show disease-modifying properties in MS (Isaacs & Lindenmann, 1957; Jacobs, O'Malley, Freeman, & Ekes, 1981). Interferons are cytokines produced by leucocytes, fibroblasts and endothelial cells in response to viral infection (González-Navajas, Lee, David, & Raz, 2012). After a decade of studies, recombinant IFN- ß -1b was FDA approved for treatment for RRMS in 1993 (McGraw & Lublin, 2013). In the years to come, IFN-ß-1a followed in different modes of application and doses. Both were also licensed for use in CIS and SPMS. Inconsistent evidence exists for IFN treatment in PPMS. Montalban and colleagues showed a slower progression and benefits in MRI for IFN-ß-1b, but a Cochrane systematic review could not verify such beneficiality and recommended a larger sample size (Montalban, 2004; Pöhlau, Hoffmann, & Harzheim, 2012; Rojas, Romano, Ciapponi, Patrucco, & Cristiano, 2010). Common side effects under IFN therapy include flu-like symptoms, injection site reactions, headache, myalgias, fever, and fatigue (Giovannoni, Southam, & Waubant, 2012).

Glatirameracetate (GA) is the second compound approved for use in RRMS and CIS. Structurally similar to the myelin basic protein, it was developed to provoke EAE in experimental animals. It turned out to ameliorate rather than aggravate disease severity (Arnon, 1996; McGraw & Lublin, 2013; Teitelbaum, Meshorer, Hirshfeld, Arnon, & Sela, 1971). It was first tested in MS patients in 1977, and in 1987 was shown to reduce relapse rates (Abramsky, Teitelbaum, & Arnon, 1977; Bornstein et al., 1987). Its mechanism of action is still not entirely known, but it has been suggested to induce a beneficial environment by shifting from pro-inflammatory Th1 cells to anti-inflammatory Th2 cells by binding to the major histocompatibility complex (MHC) II domain of MBP-recognizing antigen-presenting cells and stimulating anti-inflammatory type II monocytes, and thereby lowering production of detrimental IL-12, tumor necrosis factor (TNF) and Th17 cells (Blanchette & Neuhaus, 2008; McGraw & Lublin, 2013; Weber et al., 2007). GA has very few side effects and even a beneficial effect on fatigue (Giovannoni et al., 2012; Ziemssen, Hoffman, Apfel, & Kern, 2008).

Recently, two oral drugs have been approved for mild/moderate MS. Dimethyl fumarate is structurally related to its precursor fumaric acid, which has been used for the treatment of psoriasis since 1994 in Germany (di Nuzzo, Orlando, Nasca, & Nicoletti, 2014). Its molecular mechanism of action is based on activation of Nrf2, a leucine zipper transcription factor, which delegates the expression of antioxidant proteins and detoxification enzymes (di Nuzzo et al., 2014; Scannevin et al., 2012). Teriflunomide is the active metabolite of Leflunomide, which up to this point has been used for treatment of rheumatic arthritis (di Nuzzo et al., 2014; Killestein, Rudick, & Polman, 2011). On a molecular basis, teriflunomide inhibits the dihydroorotate dehydrogenase (DHODH), a key enzyme for pyrimidine synthesis. Normal cells have the ability to produce pyrimidines independent of DHODH and can thus salvage deoxyribonucleic acid (DNA) synthesis. However, blasting cells like inflammatory lymphocytes do not have this ability and perish. This explains the selective mechanism of action (Claussen & Korn, 2012; di Nuzzo et al., 2014).

1.6.3 Disease modifying therapy in (highly) active RRMS

The first medication for highly active MS or patients who failed first-line treatment was Natalizumab, a humanized monoclonal antibody against an integrin component (α4 domain of the very late antigen (VLA)-4). This antigen is predominantly expressed on monocytes and T-cells and facilitates the passage through the BBB (Baron, Madri, Ruddle, Hashim, & Janeway, 1993; Fernández, 2013; Léger et al., 1997). In randomized control studies, treatment with Natalizumab proved highly effective at reducing the annualized relapse rate by 68%. It also reduced progression of disability by 42% and led to fewer T2 brain lesions and 90% reduction in Gd+ lesions on MRI (Havrdova et al., 2009; D. H. Miller et al., 2003; O'Connor et al., 2011; Polman et al., 2006). These results make natalizumab a highly effective treatment option for patients with active MS. However, it can only be administered with caution due to its risk for JC virus reactivation, which can cause the often fatal progressive multifocal leukoencephalopathy (PML) (Van Assche et al., 2005).

In the past years several more highly active substances have enriched MS treatment options. Alemtuzumab, a humanized monoclonal antibody against CD52 expressed on adult lymphocytes, causes prolonged lymphocyte depletion. Formerly only applied in hairy cell leukemia it has been approved for use in highly active MS since 2014 in Germany. It reduced relapse rate and even improved the mean disability score (Coles, 2013; Coles et al., 2012).

Fingolimod is the only orally available highly active drug. As a sphingosine-1-phosphate (S1P) receptor modulator, that exhibits anti-inflammatory properties in RRMS it was even suggested to aid neuronal repair (Aktas, Küry, Kieseier, & Hartung, 2010; di Nuzzo et al., 2014). Approved by the FDA in 2010, Fingolimod influences migration of lymphocytes from the lymphatic organs via the S1P1receptor and retains certain groups of lymphocytes (i.e., central memory lymphocytes) inside secondary lymphoid organs (Cohen & Chun, 2011; di Nuzzo et al., 2014).

1.6.4 Immunosuppressive Therapy for SPMS

As shown in Table 1-3, there are still very few treatment options for the secondary progressive phase and none that can effectively slow disability progression over longer periods of time. Apart from the interferones, mitoxantrone, a synthetic antracendione derivative can act

immunosuppressively and has proven to be effective in chronic progressive MS as well as in highly active RRMS. It was shown to reduce neurological disability to a moderate extent and improve ambulatory index compared to placebo (Scott & Figgitt, 2004).

1.7 Animal models of MS

1.7.1 Experimental autoimmune encephalomyelitis

As described above, the history of MS research had a breakthrough at the time when EAE became the common model for MS (Waksman, 1999). Since MS not only has a diverse pathogenesis uniting aspects of inflammation, axon degeneration, neuronal dysfunction, demyelination and gliosis, but also a nonlinear clinical course of relapses and remissions eventually proceeding into a phase of progression, it is difficult to find a model representing all aspect of this complex disease. Although the stepping-stones had been laid much earlier, it took almost 30 years for the EAE model to be acknowledged. The idea originated in a form of paralysis affecting patients after a rabies vaccination. To clarify the cause of these incidents, the research group around Koritschoner found an inflammation of the spinal cord with the clinical correlate of paralysis in rabbits inoculated with human spinal cord homogenate (Koritschoner & Schweinburg, 1925). Only a few years later, Thomas Rivers showed an immune reaction in monkeys injected with rabbit brain tissue (Rivers, Sprunt, & Berry, 1933). This became known as the active induction of EAE. Patterson and colleagues took this experiment one step further and tried to inject lymph node cells from actively immunized animals into naive animals, with the same result. The rats developed a spinal cord inflammation as well. This was consequently termed passive induction (Paterson, 1960).

In 1947 researchers found that the use of Freund's adjuvant facilitated the immunization procedure a great deal. Instead of 30 to 100 injections over the course of several months, they were able to decrease the injections to three at weekly intervals (Kabat, Wolf, & Bezer, 1947). In addition, the introduction of pertussis toxin into the immunization regimen helped improve its effectiveness. Today, depending on the regimen, a single injection is sufficient (Levine & Sowinski, 1973). Not only the adjuvants have been experimented with, but also the spinal cord

homogenate underwent changes. It was analyzed and encephalitogenic components were identified, the MBP and the proteolipid protein (PLP) (Martenson, Deibler, & Kies, 1969; Olitsky & Tal, 1952). Almost 30 years later two additional encephalitogenic proteins were identified: myelin associated glycoprotein (MAG) and the myelin oligodendrocyte glycoprotein (MOG) (Lebar & Vincent, 1981; Linnington, Webb, & Woodhams, 1984; Poduslo, 1983; Stromnes & Goverman, 2006).

After immunization the course of the disease proceeds as follows. Peripheral CD4+ cells that have escaped the immune tolerance are activated (Seamons, Perchellet, & Goverman, 2003). It is easier for myelin-specific CD4+ T-cells to pass the BBB than for naive ones (Hickey, 1991; Wekerle, Linnington, Lassmann, & Meyermann, 1986). Once inside the CNS, they induce a neuroinflammatory response and recruit more cells. T-cells, B-cells, macrophages and plaques of demyelination can then be found. This pathophysiological picture shares many features with MS (Raine, 1997; Sospedra & Martin, 2005).

Despite the many similarities, several aspects of the EAE model are still lacking. First, the progressive phase of the disease is not sufficiently mimicked. Secondly, the role of CD8+ T-cells and B-cells has not yet been captured adequately. Thirdly, EAE lesions are most commonly found in the subpial areas of the spinal cord and the model shows fewer brain lesions than MS. Lastly, therapeutic studies have been unsuccessful due to differences in immunological reactions between humans and mice (Berer, Wekerle, & Krishnamoorthy, 2011; Huseby et al., 2001; Ransohoff, 2006, 2012; Ransohoff, Howe, & Rodriguez, 2002).

1.7.2 Other animal models of MS

Neurotropic viruses are able to cause demyelinating disease and can therefore be used in MS research, especially for understanding the mechanisms behind virally induced autoimmunity that can sustain itself in the absence of a pathogen (Bergmann, Lane, & Stohlman, 2006; Ransohoff, 2012). A favored model for oligodendrocyte depletion is toxin-mediated. Cuprizone is an agent that, when fed to mice, can block complex IV function in mitochondria and cause selective oligodendrocyte cell death with ensuing demyelination (Claudia Lucchinetti et al.,

1999; Matsushima & Morell, 2001; Ransohoff, 2012). At cessation of treatment, mechanisms of remyelination can be studied (Matsushima & Morell, 2001). Direct injection of lysophosphatidylcholine into white matter can also cause immediate demyelination followed by spontaneous remyelination over time (Blakemore, Eames, Smith, & McDonald, 1977; Blakemore & Franklin, 2008). Application of H_2O_2 or a nitric oxide (NO) donor, such as spermine NONOate, has been used to induce immediate oxidative damage and follow the cellular and subcellular processes in the absence of demyelination (Nikić et al., 2011).

A variety of models exist to study the pathogenesis of MS, each of which illustrates aspects of the disease process. However, future investigations need to be directed at the progressive stage, for which a proper model has not yet been developed (Ransohoff, 2012).

1.8 Pathology

1.8.1 Trigger factors

Given its complexity of clinical symptoms, it stands to reason that its pathogenesis is just as sophisticated. It is still the question whether MS is mainly autoimmune in origin, i. e., triggered by an external agent priming the immune system to mount an attack on the CNS, a degenerative process intrinsic to the CNS, or an interplay of both (Friese & Fugger, 2007; Mallucci, Peruzzotti-Jametti, Bernstock, & Pluchino, 2015). The two main mechanisms that can trigger such an inflammatory CNS response are thought to be molecular mimicry or bystander activation. Molecular mimicry is a process, in which the body mounts an attack against a pathogen that is similar in structure to endogenous proteins, and this attack is consequently directed against the body's own structures, i.e., myelin proteins. Bystander activation requires the activation of intrinsic CNS antigen-presenting cells, which in turn prime T-cells to initiate an autoimmune response (Fujinami & Oldstone, 1985; Libbey, McCoy, & Fujinami, 2007; Münz, Lünemann, Getts, & Miller, 2009; Sospedra & Martin, 2005; Wucherpfennig & Strominger, 1995).

Several possible targets of the autoimmune response have been discovered. Myelin basic protein (MBP), myelin oligodendrocyte protein (MOG), myelin-associated glycoprotein (MAG), proteolipid protein (PLP), and 2' ,3' -cyclic- nucleotide 3' -phosphodiesterase (CNPase) are the most common ones (Mallucci et al., 2015; McCarthy, Richards, & Miller, 2012; Sospedra & Martin, 2005).

MBP is one of the key elements that build up the myelin sheath. It has been a focus of MS research since it was found to induce EAE (Belogurov et al., 2014; Karin, Mitchell, Brocke, Ling, & Steinman, 1994; Petry et al., 2000; Steinman, 1995). Interestingly, it shares the MHC class II and the T cell receptor binding motifs with several viruses, most notably EBV, which has been found in lesions and in serum of MS patients (Ascherio & Munger, 2007; Mallucci et al., 2015; Peferoen et al., 2010; Sargsyan et al., 2010; Serafini et al., 2007; Willis et al., 2009). Furthermore, the risk of developing MS is greater after primary EBV infection and dependent on the antibody titer (Levin, Munger, O'Reilly, Falk, & Ascherio, 2010; K. L. Munger, Levin, O'Reilly, Falk, & Ascherio, 2011). Nissen and colleagues have shown that human endogenous retroviruses can play a role in the activation of the immune system, perhaps by producing proteins that can start a process similar to infection (Nissen et al., 2013). This might also explain the positive impact on disease through antiretroviral drugs (Maruszak, Brew, Giovannoni, & Gold, 2011; Nexø, Pedersen, Sørensen, & Koch-Henriksen, 2013).

1.8.2 Immunological mechanisms behind lesion formation

Different hypotheses on the mechanism of lesion formation have been postulated. The classical hypothesis proposes that the initial damage arises from the adaptive immune system and is followed and augmented by actions of the innate immune system. Peripheral T-cells with encephalitogenic potential are activated by mechanisms of molecular mimicry, bystander activation or cross reactivity (Sospedra & Martin, 2005; Wucherpfennig & Strominger, 1995). Although the location for this activation is not yet known, suggested are the gut, lungs or in the skin (Hemmer et al., 2015). They migrate towards the lymph nodes from which a small subset invades the CNS and initiates the disease. Plasma cells can produce antibodies directed against myelin and glia cells, whereas T-cells secrete inflammatory cytokines to promote glial damage

and increase permeability of the BBB. Disruption of the BBB is followed by an influx of monocytes and lymphocytes (Hemmer et al., 2015).

The alternative hypothesis involves intrinsic damage to the CNS due to oligodendrocyte dysfunction or extrinsic damage caused by a virus or other pathogen. This is followed by initial activation of the innate immune system of the brain, which implies a recruitment of microglia (Mallucci et al., 2015). This initial inflammation might prompt a leakage of proteins from the CNS into the CSF through the BBB. These proteins accumulate in draining lymph nodes, where they induce maturation of peripheral B-lymphocytes reactive against CNS proteins (Stern et al., 2014). Integrins are upregulated on lymphocyte surfaces to facilitate binding to vascular cells adhesion molecules (VCAMs) on the endothelial cells. Finally the lymphocytes migrate across the BBB and into the CNS (Mallucci et al., 2015; Ransohoff & Engelhardt, 2012; Wekerle et al., 1986). Once inside they secrete pro-inflammatory molecules such as IFNγ and osteopontin (OPN), which is followed by a massive infiltration of microglia and macrophages to the area of inflammation (Chabas et al., 2001; Mallucci et al., 2015). Macrophages in turn release IL-8, CXC-ligand 10, macrophages inflammatory proteins 1a/b and other cytokines to recruit more intracerebral T-cells, macrophages and dendritic cells (Aloisi, 2001; Mallucci et al., 2015; McMahon, Bailey, Castenada, Waldner, & Miller, 2005; Owens, Gilden, Burgoon, Yu, & Bennett, 2011).

1.8.3 White Matter Lesion classification

In an international multicenter study, Lucchinetti and colleagues collected and examined lesion specimen from biopsies and autopsies in order to classify demyelinating lesions in MS (C. Lucchinetti et al., 2000). They found four distinctly different types. All lesions showed infiltrates of T lymphocytes and macrophages, diffuse immunoglobin (Ig) reactivity and reactive astrogliosis as a marker for BBB breakdown.

Type I and Type II showed a sharply demarcated perivenous T-cell and macrophage infiltration. Although similarly constructed, pattern II lesions included depositions of IgGs and complement at the active rim of the plaque, suggesting a complement mediated myelin degradation and an

involvement of antibodies. In pattern I the degradation was mainly initiated by macrophage products. In pattern III lesions, a T-lymphocyte dominant infiltrate could be detected within a vaguely defined plaque area. The rim was composed of concentric layers of demyelinated and myelinated tissue with prominent loss of oligodendrocytes. Oligodendrocytes could not be detected in the center. Interestingly, only the MAG myelin protein was predominantly lost. There were no remyelinated shadow plaques.

Pattern IV infiltrates are also dominated by T lymphocytes and macrophages without Ig and complement depositions. A small zone of oligodendrocyte destruction was found at the rim of the active demyelination in the periplaque white matter.

Interestingly, all patients examined at autopsy revealed only one plaque pattern. The most commonly found was pattern II, followed by III, I and IV. Pattern III was seen almost exclusively in acute MS, whereas patterns II and I were found in other clinical courses as well. Pattern III also indicated a higher relative risk for monophasic disease. Pattern IV was seen in PPMS patients with cognitive, brainstem and cerebellar impairments. Pattern I and II lesions are those represented by the established animal models.

The different pathologies behind those patterns lead to the assumption of different origins for disease pathology. Pattern I and II might be the result of an autoimmune attack due to the appearance of antibodies, whereas pattern III might be a virus-induced dystrophy of oligodendrocytes and demyelination. Although the patients at autopsy only revealed a homogenous pattern type, it is not clear whether the type remains the same or undergoes changes throughout disease progression (C. Lucchinetti et al., 2000). Recent evidence suggested however that patients retained their pattern throughout the disease until all lesions assume the same morphology of chronically inactive lesions (Metz et al., 2014). Keegan et al. postulated that patients with pattern II lesions, due to their antibody content, are more susceptible to plasmapheresis (Keegan et al., 2005).

1.8.4 Gray matter lesions

In 1962 Brownell and Hughes first reported that 26% of MS lesions are found in the gray matter or at the border to the white matter (Brownell & Hughes, 1962; Mallucci et al., 2015). Only in the past years has gray matter damage come into the focus as a separate pathologic hallmark of MS. Gray matter lesions are located in the cortex, most often subpially in close proximity to the meninges. B-cells have been found to form aggregates in the meninges of SPMS patients. Supposedly these cells greatly influence gray matter pathology by constantly releasing cytokines that sustain a chronic activation of resident microglia (Choi et al., 2012; Peterson, Bö, Mörk, Chang, & Trapp, 2001). B-cell accumulations have more often been found in patients with a worse clinical disease course and more prominent cortical atrophy (Howell et al., 2011; Magliozzi et al., 2007). A great proportion of gray matter lesions are associated with extensive neuronal and synaptic loss (Wegner, Esiri, Chance, Palace, & Matthews, 2006). Not surprisingly, gray matter pathology is thought to be a main factor behind cognitive decline and physical disability (Calabrese, Favaretto, Martini, & Gallo, 2013).

1.8.5 Demyelination

In the CNS, oligodendrocytes produce the myelin layer, which wraps around the axon and provides trophic support, helps sustain homeostasis and enables saltatory signal conduction (Waxman & Ritchie, 1993). Demyelination can be initiated by different mechanisms. First, an autoimmune attack on myelin proteins such as PLP, MOG or MBP by autoantibodies or cytotoxic factors (TNFα, NO, IFNγ) secreted from macrophages can cause direct damage of the myelin sheath (Bitsch, Schuchardt, Bunkowski, Kuhlmann, & Brück, 2000; Genain, Cannella, Hauser, & Raine, 1999; C. Lucchinetti et al., 2000). Second, indirect myelin breakdown can be caused by damage to oligodendrocytes, which is a prominent feature of lesion patterns III and IV (C. Lucchinetti et al., 2000).

1.8.6 Remyelination

Soon after the initial inflammatory burst has come to an end, oligodendrocyte precursor cells (OPCs) and oligodendrocytes initiate remyelination (Keirstead & Blakemore, 1999; Smith, Blakemore, & McDonald, 1979). With each cycle of demyelination and remyelination, the myelin sheath becomes thinner and shorter (Ludwin & Maitland, 1984). Mechanistically, OPCs are drawn to the demyelinated lesion by growth factors and activation of transcription factors. When they have established contact to the axon, they begin expressing myelin-specific genes and finally cover the denuded axon membrane (Mallucci et al., 2015). In chronic lesions, this process is rarely seen, perhaps due to the milieu enriched with substances suppressing OPC differentiation and recruitment (Mallucci et al., 2015; Tepavčević et al., 2014). New therapeutic strategies are aimed at enhancing remyelination, such as the retinoic X receptor agonist 9-cis-retinoic acid (Huang et al., 2011; Huang & Franklin, 2012).

1.8.7 T-cells and B-cells

Multiple sclerosis is a T-cell dominated disease. Autoreactive CD4+ T-cells directed against MBP and MOG can induce EAE and likely act as disease initiator in MS as well (Hemmer et al., 2015; Mendel, Kerlero de Rosbo, & Ben-Nun, 1995). The Th1 subtype secretes IFNγ and produces an inflammation mainly confined to the spinal cord, whereas Th17 cells mainly release IL17 and induce an inflammation of the brain stem, cerebrum and cerebellum (Hemmer et al., 2015; Legroux & Arbour, 2015). Presumably CD4+ T-cells are primed in the periphery, the location of which remains elusive (Hemmer et al., 2015). For these cells to enter the CNS they need to possess a distinct combination of surface receptors and integrins to dock onto the vascular wall and transmigrate through the BBB. A role for CD8+ T-cells in inflammation and demyelination has been proposed as well (Bitsch et al., 2000). CD8+ T-cells even outnumber CD4+ T-cells in postmortem studies in brain of MS patients (Booss, Esiri, Tourtellotte, & Mason, 1983). CD8+ T-cells are also found predominantly in gray matter lesions (Calabrese et al., 2015). MHC class I molecules, the docking receptors on parenchymal nucleated cells for CD8+ T-cells, are found upregulated on neurons reacting to external or internal danger signals (Siffrin, Vogt, Radbruch, Nitsch, & Zipp, 2010). Cytotoxic T-cells are found in culture and in vivo studies in contact with

degenerating axons, which suggests their role in axonal injury (Medana, Martinic, Wekerle, & Neumann, 2001; Neumann, Medana, Bauer, & Lassmann, 2002). In imaging studies, T-cells labeled with superparamagnetic iron oxide nanoparticles (ferumoxides) have been shown to migrate at disease onset (Anderson et al., 2004).

Although B-cells are not the main contributors in MS lesions, they nevertheless have been ascribed a role in mediating gray matter damage, especially in cortical lesions (Mallucci et al., 2015). As presenters of antigens they might play a role in exacerbating demyelination, in line with the finding that OCBs in the CSF of CIS patients suggest an increased risk for conversion (Dobson, Ramagopalan, Davis, & Giovannoni, 2013). The effectiveness of Rituximab, a CD20 antibody directed against a surface protein of B-cells, in highly active cases supports the role of B-cells in MS (Hauser et al., 2008).

1.8.8 Microglia and Macrophages

Microglia can adapt various activational states depending on the milieu, in which they are situated (Orihuela, McPherson, & Harry, 2015). They sustain tissue homeostasis, phagocytose debris, maintain synapse integrity, promote neurogenesis, secrete essential growth factors and support neuronal integrity (Graeber, 2010; Kettenmann, Hanisch, Noda, & Verkhratsky, 2011; Murabe & Sano, 1982; Nimmerjahn, Kirchhoff, & Helmchen, 2005; Ransohoff & Perry, 2009; Tremblay et al., 2011). Their even distribution allows them to react immediately to any change in environment (Tremblay et al., 2011). Once activated, they retract their processes, adopt an ameboid shape and migrate towards the site of damage (Davalos et al., 2005; Graeber, 2010; Stence, Waite, & Dailey, 2001). Two distinct phenotypes have been distinguished: the classically activated M1 microglia causing tissue damage by release of neurotoxic substances and the alternatively activated M2 microglia, that have been shown to promote tissue repair and keep inflammation at bay (Cherry, Olschowka, & O'Banion, 2014; Orihuela et al., 2015). Their versatile profiles point to a profound role in the pathology of MS, not only by abandoning their surveillance and supporting role and adopting a possibly detrimental one, but also by adopting a protective phenotype, which might aid in remyelination and tissue repair. In MS, macrophages and microglia represent the greatest proportion of inflammatory cells in a lesion

(Lucchinetti et al., 2000). They secrete inflammatory mediators, cytokines, reactive oxygen and nitrogen species (ROS and RNS), which not only damage the myelin sheath but also the axons beneath, by creating oxidative stress (Fischer et al., 2012; H. Lassmann, 2003; Nikić et al., 2011). It is difficult to discern whether macrophages in a lesion are of monocytic or microglial origin, owing to their similar capacities and immunohistochemical markers. However, Yamasaki and colleagues have used distinct gene-expression patterns and morphological features to differentiate these and were able to demonstrate that monocyte-derived macrophages initiate demyelination at the nodes of Ranvier, whereas macrophages of microglial origin promote repair processes and clear debris (Yamasaki et al., 2014). This emphasized the dual role of inflammation in inflammatory CNS diseases such as MS. With cumulating evidence, inflammation cannot solely be viewed as detrimental but also as beneficial in promoting remyelination and tissue repair as well. The goal in the search for new therapeutic strategies should possibly be to modulate inflammation rather than to eradicate it.

1.9 Axonal pathology

1.9.1 Hallmarks of axonal pathology

Axonal injury are pathologic changes of axonal structures that if uninhibited amount to axonal death (Harris & Sloane, 2012). With improved staining methods for degenerating axons, such as the amyloid precursor protein (APP), Ferguson showed that axonal injury occurs in acute plaques and at the active rim of chronic plaques in MS, namely in areas with high inflammation. Transport deficits lead to an accumulation of intracellular substances and distensions along the axon (spheroids) appear (Ferguson, Matyszak, Esiri, & Perry, 1997). If those focal swellings continue to grow and reach a threshold, the axon will begin to segment and large bulb-like formations will mark the point of transection (Lovas, Szilágyi, Majtényi, Palkovits, & Komoly, 2000; Peterson et al., 2001; B. D. Trapp et al., 1998). Those swellings can be visualized by staining for APP, which is an integral membrane protein found in neuronal synapses associated with the endosomal/lysosomal systems, synapse formation and neuronal plasticity. It is carried by fast axonal transport and can only be detected when transport is disturbed (Ferreira,

Caceres, & Kosik, 1993; Haass, Koo, Mellon, Hung, & Selkoe, 1992; Harris & Sloane, 2012; Koo, Park, & Selkoe, 1993; Sherriff, Bridges, Gentleman, Sivaloganathan, & Wilson, 1994). A second option is to stain for non-phosphorylated neurofilament (B. D. Trapp et al., 1998). Neurofilaments, essential for structure and shape of an axon, are usually phosphorylated in order to increase interneurofilament spacing, which leads to larger caliber axons, and thus increases conduction velocity and enables fast axonal transport (Dutta et al., 2006). In demyelinated axons, neurofilaments lie free to be dephosphorylated, so that spacing, diameter, conduction and transport subsequently decrease (de Waegh, Lee, & Brady, 1992; Harris & Sloane, 2012; Hsieh, Crawford, Bouldin, & Griffin, 1993). Several groups reported reduced axon density, prevalence of small caliber axons and of axon loss upon autopsy (Ganter, Prince, & Esiri, 1999; Klaver et al., 2015; Lovas et al., 2000).

Today, axonal loss is considered to represent the major factor behind permanent disability and the secondary progressive course (Ferguson et al., 1997; Harris & Sloane, 2012).

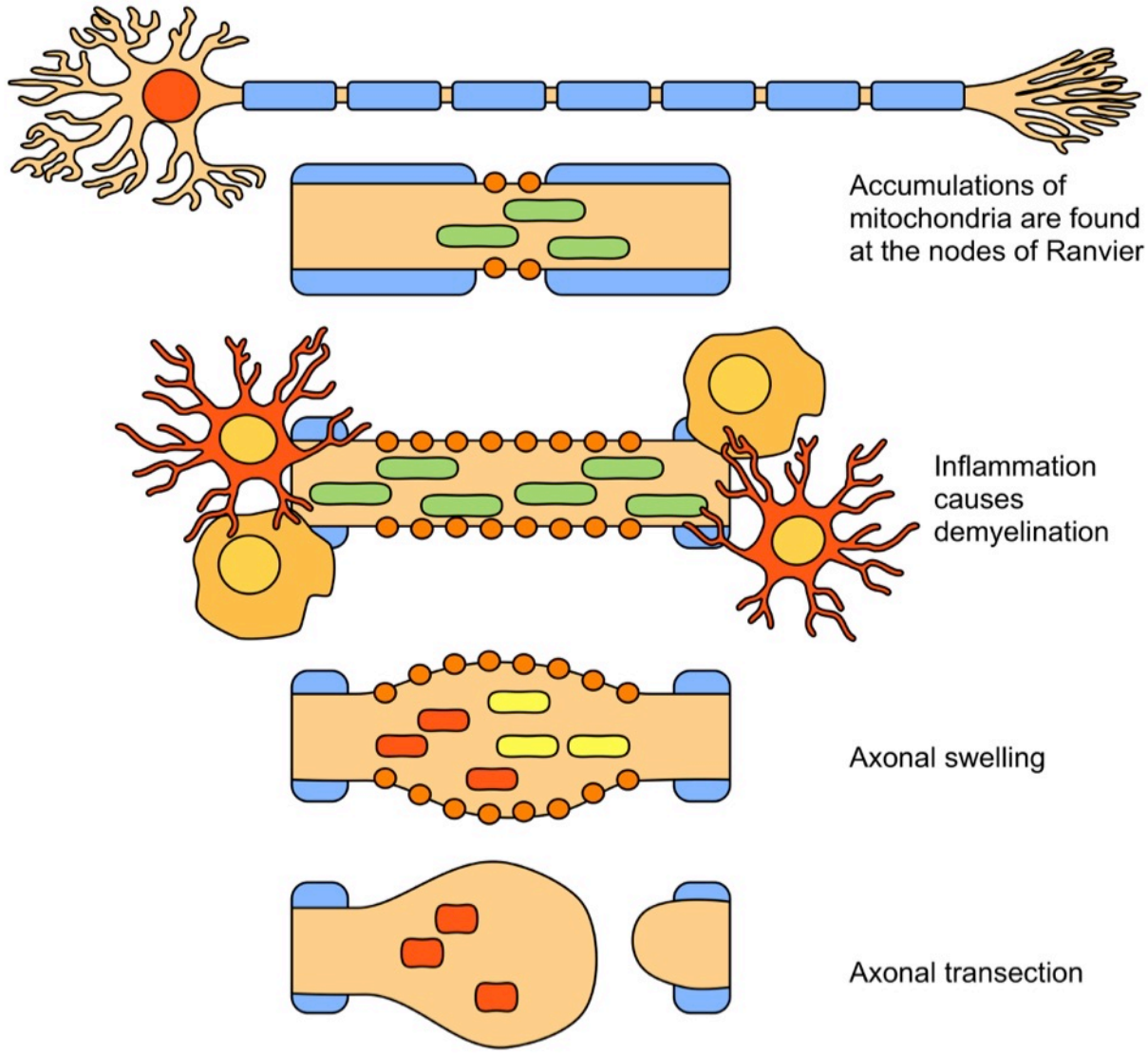


Fig. 1-3 Axonal degeneration

Demyelination caused by inflammation leads to redistribution of Nav1.6 channels (orange) along the denuded membrane, and ROS and NO induce mitochondrial pathology and interrupt axonal transport. This leads to swelling and energy failure and culminates in axonal transection.

1.9.2 Causes of axonal pathology

There are several possible mechanisms that can contribute to axonal swelling and transection.

1. Demyelination and oligodendrocyte death
2. Inflammation
3. Metabolic imbalance
4. Dissolution of the cytoskeleton
5. Wallerian degeneration

First of all demyelination, as one of the hallmarks of MS, is considered to be a major contributing factor in axonal injury. This is not only the case because myelin is the basis for saltatory signal conduction and the energy failure that ensues with demyelination. The relationship between axon and myelin goes far beyond facilitating the flow of electrical currents. The ensheathing myelin has been shown to interact with the encapsulated neuron by secreting and absorbing transmitters, toxic substances, misfolded protein aggregates, neurofilaments and even mitochondrial parts and thereby relieving the axon of its metabolic burden (Brahic & Roussarie, 2009). Most of our knowledge about this interaction stems from the peripheral nervous system (PNS), where Schwann cells have been shown to deliver small exosomes to axons to locally support regeneration after nerve damage (Lopez-Verrilli, Picou, & Court, 2013). This relationship might on the other hand also be disadvantageous as other studies showed that following axonal injury an upregulation of signaling cascades in Schwann cells might lead to myelin fragmentation and spread of the injury (Simons, Misgeld, & Kerschensteiner, 2014)

When the myelin sheath breaks down due to an autoimmune attack, saltatory conduction will first be replaced by continuous conduction, which is not only slower but also vastly increases the energy demand. This is due to a diffuse expression of voltage gated Nav1.2 and Nav1.6 channels and adenosine triphosphate (ATP) dependent ion-transporters to counteract the influx of positively charged ions (Craner et al., 2004). These channels were found in close proximity to βAPP, a marker for axonal injury. Due to the increased energy demand

mitochondria are redirected to demyelinated parts of the axon, where mitochondrial content is found to be elevated (Zambonin et al., 2011).

Meanwhile, on the other side of the axonal membrane, inflammatory cells can directly attack axons. As reported by several studies, there appears to be a direct correlation between axonal injury and inflammation (Bitsch et al., 2000). Inflammatory cells release a toxic cocktail of NO, TNFα, and IFNγ, which can cause further damage to the BBB, paving the way for more inflammatory cells into the CNS (Andrews et al., 2006). NO interferes with mitochondrial metabolism, specifically inhibits complex I and IV of the respiratory chain on the one hand, and causes conduction failure and structural damage in electrically active axons on the other hand (Bolanos, Almeida, & Stewart, n.d.; D. Mahad, Ziabreva, Lassmann, & Turnbull, 2008; Smith, Kapoor, Hall, & Davies, 2001). Moreover, NO and its reaction products can modulate ion channels, receptors, and glycolytic enzymes, thereby increasing the strain on mitochondrial metabolism (Pacher, Beckman, & Liaudet, 2007). TNFα and IFNγ are cytokines that attract more immune cells to the area and can aggravate inflammation (Fraga-Silva et al., 2015).

The previously mentioned factors lead to an imbalance in energy metabolism. Elevated intracellular Na^+ leads to a reversal in function of the Na^+/Ca^{2+}exchanger and is therefore replaced by Ca^{2+}. More Ca^{2+} is released from axonal stores through glutamate signaling via GluR6 and by insertion of the pore-forming subunit of (N)-type voltage gated Ca^{2+} channels into the demyelinated membrane (Fern, Ransom, & Waxman, 1995; Ouardouz et al., 2003, 2009; Ouardouz, Malek, Coderre, & Stys, 2006). GluR6 receptors were found to increase intracellular NO, which was shown to mediate the Ca^{2+} increase (Ouardouz et al., 2009).

There are several ways, in which elevated Ca^{2+} can amplify or even induce axonal injury. First, increased axonal Ca^{2+} leads to activation of degradative enzymes such as phosphatases. These dephosphorylate neurofilaments, which, as described above, can lead to reduced velocity of fast axonal transport including mitochondrial transport to and from the lesion (Strack, Westphal, Colbran, Ebner, & Wadzinski, 1997). Second, Ca^{2+} also activates the neural proteinase calpain, which is able to degrade all major myelin proteins (Shields, Schaecher, Saido, & Banik, 1999) as well as the axonal cytoskeleton. Third, Ca^{2+} excess can destabilize mitochondria (Horng, 2014; Witte, Mahad, Lassmann, & van Horssen, 2014). Mitochondrial function fails at

this point for a number of reasons. Primarily, due to increased energy demands, the mitochondria in the lesion fail to power fast axonal transport and recruit more mitochondria into the lesion area. Then, increased ROS production by inflammatory cells and mitochondria due to inhibition of respiratory chain function leads to even more oxidative damage to the mitochondrial machinery. Lastly, the failure of mitochondria to buffer calcium excess leads to activation of degradative enzymes. These processes create a self-perpetuating cycle of energy deficiency, metabolic demands and mitochondrial and axonal dysfunction that ultimately leads to axonal transection (Bruce D. Trapp & Stys, 2009; Witte et al., 2014). Wallerian degeneration sets in when the distal process is separated from the cell body and degenerates anterogradely (Dziedzic et al., 2010). Especially in normal-appearing white matter (NAWM) damage, it has been proposed to play an important role (Evangelou et al., 2000).

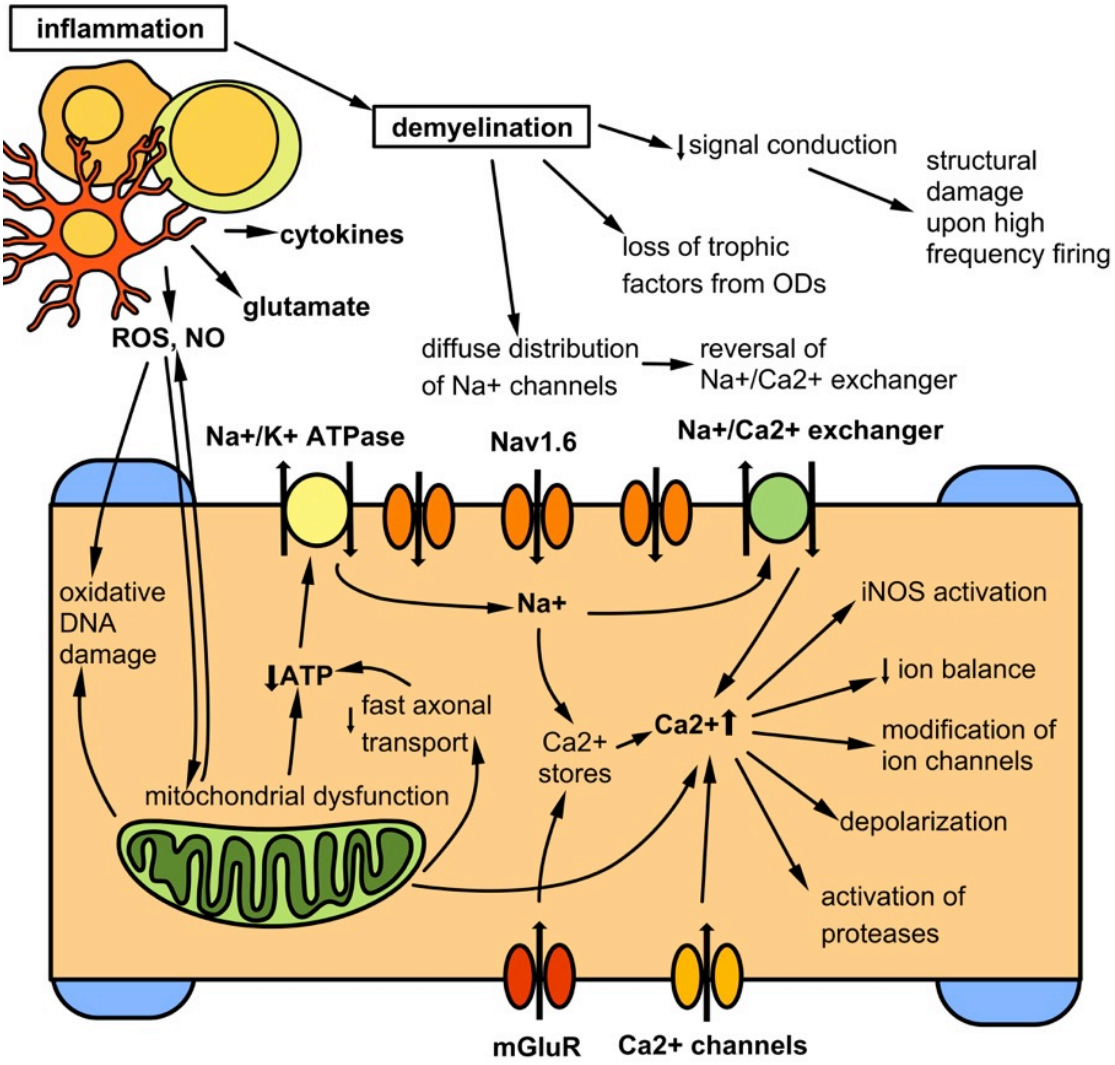


Fig. 1-4 Processes leading to axonal degeneration

As inflammatory cells (microglia in red, T-cells in green, macrophages in orange) invade the CNS, they release cytokines, glutamate, and ROS and NO and thereby create a neurotoxic milieu. ROS and NO damage DNA and inhibit mitochondrial respiration. In turn, mitochondrial dysfunction causes energy failure of the axon, ATPases cease to work and fast axonal transport is arrested. Concurrent demyelination leads to loss of saltatory conduction, diffuse distribution of Na^+ channels along the denuded membrane and loss of trophic support from the oligodendrocyte. Na^+ accumulates intracellularly, which ultimately reverts the function of the Na^+/Ca^{2+}-exchanger and leads to an increase in intracellular Ca^{2+}. Other mechanisms contributing to the increase in Ca^{2+} load are mitochondrial release of Ca^{2+}, Ca^{2+} release from intracellular stores through activation of glutamate receptors and Na^+ channels, and through Ca^{2+} channels built into the membrane. Ca^{2+} activates proteases, lipases, and inducible nitric oxide synthase and modifies ion channels, which culminate to axonal transection and/or neuronal death (adapted from Ciccarelli et al., 2014; Waxman, 2006). Permission was obtained from Elsevier and Nature Publishing Group.

In summary, axonal damage has multiple origins and may be a result of interplay between demyelination, mitochondrial changes, distal insults to the neuronal soma or proximal axon districts, local meningeal inflammation, and microglial activation, which can occur singly or as additional factors in the process of axonal pathology.

1.10 Mitochondrial pathology

1.10.1 Mitochondrial respiratory chain – a source of oxidative stress

Mitochondria are indispensable for proper function and integrity of the neuron (Nicholls & Budd, 2000). By producing ATP, they maintain diverse neuronal functions such as axonal transport, assembly of the cytoskeleton, and fatty acid oxidation. They also sustain synaptic function, i.e. vesicle exocytosis and Ca^{2+} buffering, facilitate neuronal conduction, regulate apoptosis, and are potent producers of ROS (D. C. Chan, 2006; Li, Okamoto, Hayashi, & Sheng, 2004; Misgeld, Kerschensteiner, Bareyre, Burgess, & Lichtman, 2007; Sheng & Cai, 2012). As displayed in Fig 1-5 electrons are being transferred through a chain of enzymes (complexes I-IV), while protons (H^+) are being pumped from the matrix into the inner membrane space thereby generating a transmembranous gradient, which is ultimately being used to produce ATP (Jin et al., 2014). Through leakage of electrons reactive oxygen species are created (i.e. oxygen superoxide). To counteract oxidative reactions with biomolecules the mitochondrion contains an arsenal of antioxidative reactants, such as glutathione peroxidase, catalase, and thioredoxin reductase (Hans Lassmann & van Horssen, 2011). At physiological levels ROS are involved in signaling cascades (Jin et al., 2014). In pathological conditions they are often produced excessively not only by the mitochondrion but also by external sources such as microglia and macrophages. This leads to an imbalance with a relative lack of anti-oxidative agents.

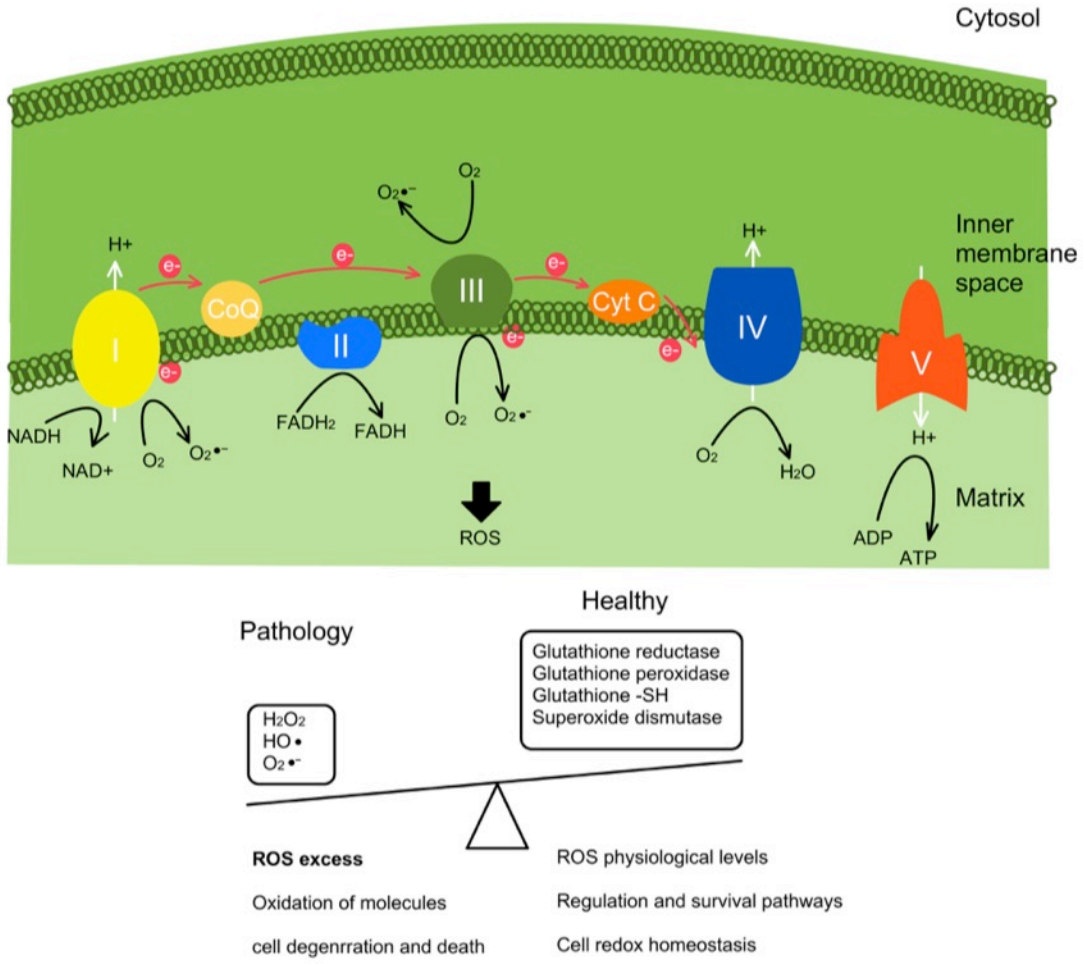


Fig. 1-5 Mitochondrial ROS production

While electrons are being transferred from complex I onto complex IV protons are channeled into the inner membrane space. Across the inner membrane a gradient is created, which forces protons back into the matrix through complex V, where the energy is used to generate ATP from ADP. The end products are water and carbondioxide. If there is a shortage of reductive compounds and due to electron leakage throughout the process, ROS are generated. ROS at a healthy level help regulate survival pathways and contribute to cell homeostasis. An excess on the other hand can lead to cell degeneration and apoptosis.

Adapted from (Jin et al., 2014; Moreira et al., 2010). Rights obtained from Elsevier.

1.10.2 Mitochondrial transport: general

Owing to their complex polarized structure and unique energy requirements, neurons pose an especially difficult challenge for mitochondrial distribution (Hollenbeck & Saxton, 2005; Sheng & Cai, 2012). Under normal conditions, mitochondrial distribution is regulated by the energy demands of the various cellular compartments, accounting for the unequal and fluctuating

allocation of mitochondria (Fabricius, Berthold, & Rydmark, 1993; Hollenbeck & Saxton, 2005; Zhang, Ho, Kintner, Sun, & Chiu, 2010). High ATP consumption and therefore mitochondrial accumulations are found in the nodes of Ranvier, active synapses, myelination boundaries, active growth cones and newly forming collaterals (Amiri & Hollenbeck, 2008; Berthold, Fabricius, Rydmark, & Andersén, 1993; Bristow, Griffiths, Andrews, Johnson, & Turnbull, 2002; Li et al., 2004; Waxman & Ritchie, 1993; Wong-Riley & Welt, 1980). During an acute demand such as the process of forming new synapses or growing new collaterals, mitochondria are often initially merely relocated within the distal axon segment (Amiri & Hollenbeck, 2008). However, a constant supply of entire mitochondria and repair parts can only be maintained by sending material from the soma.

As the only cell organelle to contain non-nuclear DNA, mitochondria have a unique position. Mitochondrial DNA (mtDNA) codes for 22 transfer RNAs and 13 polypeptides essential to oxidative phosphorylation (Schon, DiMauro, & Hirano, 2012). Most genes for the mitochondrial assembly are encoded in the nucleus. Consequently the overall majority of mitochondria will be produced and assembled on site and transported to the distal parts of the axon. Interestingly, several groups have also discussed the possibility of a distal protein synthesis, mitochondrial assembly, and mtDNA replication (Magnusson, Orth, Lestienne, & Taanman, 2003; Twiss & van Minnen, 2006). However either way, distal mitochondrial supply requires a sophisticated transport system (Sheng & Cai, 2012).

Axonal transport is differentiated into fast and slow, anterograde (towards the periphery) and retrograde (towards the soma). With the possibility to visualize and to observe *in-vivo* mitochondrial trafficking through the axon, mitochondrial migration patterns could be followed in the healthy and diseased state (Misgeld, Kerschensteiner, et al., 2007). In time-lapse recordings of acute explants of peripheral nerves Misgeld and Kerschensteiner identified three categories of mitochondria: immobile, anterogradely and retrogradely traveling mitochondria. The immobile mitochondria constituted the largest population with 87% and they were approximately double the size of the mobile fraction (3.00µm). Those moving anterogradely, represented two thirds of the mobile fraction, were slightly shorter and slower than the retrogradely transported population. Mitochondria generally traveled with occasional pauses

(Misgeld, Kerschensteiner, et al., 2007). In cultured neurons they are moved from two to five μm per second (Brady, 1991, 1993).

A complex interplay between motor proteins, anchor proteins, sensors, the transported organelle and the scaffolding along which the organelle is guided, allows for a quick switch between moving and arresting, direction and speed (Chada & Hollenbeck, 2004; Hollenbeck & Saxton, 2005).

1.10.3 Mitochondrial transport: equipment

Depending on the direction, destination and cargo there exist different combinations of motor and anchor proteins. Anterograde transport is primarily steered by motor proteins from the kinesin family (N. Hirokawa et al., 1991). The dynein family is mostly responsible for retrograde transport (Pilling, Horiuchi, Lively, & Saxton, 2006; Varadi et al., 2004). Myosin, a third motor protein that transports its cargo along actin filaments, is responsible for short distance transport in presynaptic terminals, growth cones and dendritic spines (Sheng & Cai, 2012). Miro and Milton are two adaptor proteins that bind kinesin 1 to the mitochondrion.

Kinesin 1, the motor for long distance anterograde mitochondrial transport, typically consist of two kinesin 1 family 5 (KIF5) heavy chains and two kinesin light chains (Nobutaka Hirokawa, Niwa, & Tanaka, 2010). It was first isolated from axoplasm of the squid giant axon and found to transport glass beads along microtubules (Vale, Reese, & Sheetz, 1985). The heavy chains contain the ATP hydrolyzing units that provide the motor force and the light chains together with the carboxy-end of the heavy chains recognize the cargo (Nobutaka Hirokawa et al., 2010). There are six kinesin families that participate in organelle transport in axons, two of which, especially kinesin 1 and 3, are responsible for mitochondrial transport (Hollenbeck & Saxton, 2005). Of the three isoforms of kinesin 1 KIF5A, KIF5B, and KIF5C, KIF5A and C are expressed only in neurons and KIF5B is ubiquitous (Nobutaka Hirokawa et al., 2010; M.-Y. Lin & Sheng, 2015). KIF1B-α and KLP6 are the mitochondria-transporting isoforms of the kinesin 3 family (M.-Y. Lin & Sheng, 2015).

Mutations to the heavy chain of kinesin can cause lower sodium channel activity, fewer action potentials, less neurotransmitter release on a physiological basis and on a molecular basis

reduces anterograde and retrograde transport, mitochondrial density and number of synaptic boutons (Hurd & Saxton, 1996; M.-Y. Lin & Sheng, 2015). In KIF1A knock-out mice the number of axonal branches was decreased, while the number of dendrites was not significantly different, and mitochondrial transport velocity was decreased as well (Karle, Möckel, Reid, & Schöls, 2012). Mutations of kinesin 3 family members mainly alter mitochondrial distribution (M.-Y. Lin & Sheng, 2015; Tanaka, Sugiura, Ichishita, Mihara, & Oka, 2011).

Cytoplasmic dynein is a large protein complex consisting of two heavy chains with ATP hydrolyzing activity, responsible for mobility, and several intermediate chains, intermediate light chains and light chains for cargo recognition (Nobutaka Hirokawa et al., 2010; Karki & Holzbaur, 1999). Unlike kinesins the genetic variety for the heavy chains in dynein is limited, however due to the composition of the accessory parts a variety of binding options can be achieved (Hollenbeck & Saxton, 2005).

In order to link the cargo to kinesin 1, anchor proteins are needed. Miro (mitochondrial Rho GTPase) connects to the outer membrane of the mitochondrion consisting of two GTPase domains connected by four EF hands, Ca^{2+}-binding domains (Reis, Fransson, & Aspenström, 2009; Schwarz, 2013). Milton forms the link between Miro and KIF5 (Reis et al., 2009). The coiled-coil region of the N-terminal connects with the kinesin or dynein/dynactin complex and the C-terminal links with Miro (van Spronsen et al., 2013). Together they form the anterograde mitochondrial transport unit. Mutation in either adaptor resulted in transport deficits. dMiro mutants showed depleted presynaptic terminals and a complete loss of Miro1 resulted in a mitochondrial loss in the long spinal tracts (Górska-Andrzejak et al., 2003; Guo et al., 2005; Sheng & Cai, 2012). Loss-of-function mutations of Miros N- terminal of the GTPase domains caused premature lethality and showed mitochondrial accumulations in the soma of neurons, as well as reduced mobility of the motor proteins kinesin and dynein and mitochondrial fragmentation, while the C-terminal mutation merely affected mobility of dynein (Babic et al., 2015). Mutations of the Milton gene caused a lack of mitochondria within axons and synapses (Stowers, Megeath, Górska-Andrzejak, Meinertzhagen, & Schwarz, 2002). Recently trafficking kinesin proteins (TRAK) have been identified as a mammalian homologue of Milton, therefore presenting another link between kinesin and Miro (Brickley & Stephenson, 2011; MacAskill,

Brickley, Stephenson, & Kittler, 2009). TRAK1 interacts with kinesin and dynein and is found in axons, while TRAK2 primarily binds to dynein/dynactin and steers mitochondria into dendrites (van Spronsen et al., 2013). Syntabulin appears to be an adaptor protein, mainly responsible for anterograde transport. It colocalizes with mitochondria in axons, binds directly to KIF5 and enables its recruitment to mitochondria (Cai, Gerwin, & Sheng, 2005; M.-Y. Lin & Sheng, 2015).

For dynein several adaptor proteins have been suggested. Dynactin for instance binds to dynein and mitochondria, however it promotes bidirectional motility associating with KIF5 as well (Pilling et al., 2006). Miro has been postulated to play a role in retrograde transport as well (Russo et al., 2009). Since the inhibition of kinesin-1 reduced anterograde and retrograde transport, a role in retrograde transport for kinesin was suggested as well (Pilling et al., 2006). Most likely the roles of the motor proteins are even more intertwined than has been assumed. Anterograde and retrograde transport are probably regulated through a complex interaction of various motor proteins and adaptors (Sheng & Cai, 2012).

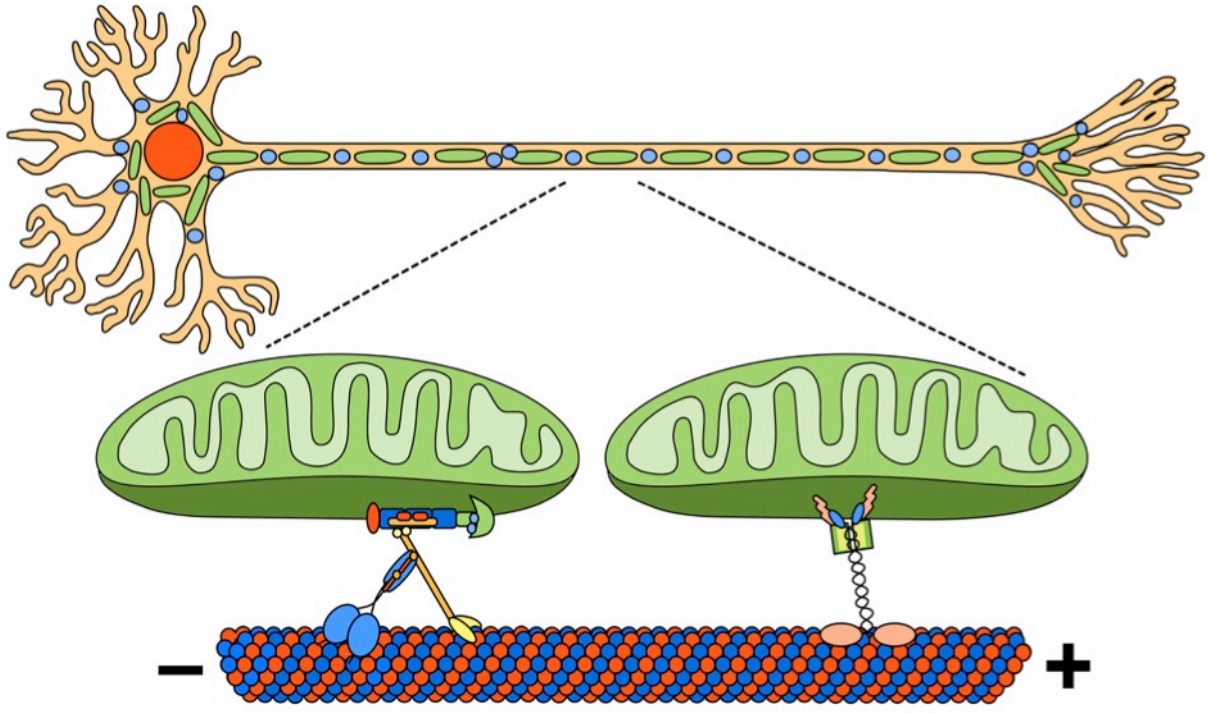


Fig. 1-6 Axonal transport of mitochondria

Mitochondria and other cargoes bind to kinesin (right) for anterograde transport and dynein (left) for retrograde transport. Kinesin consist of two heavy chains with the motor unit and two light chains docking onto the cargo. Dynein forms a complex with dynactin and consists of two heavy chains and several light and intermediate chains. Dysfunction in either part of the system can lead to undersupply

of the synapse with cargoes or maldistribution of mitochondria along the axon. Adapted from (De Vos, Grierson, Ackerley, & Miller, 2008). Permission not required.

1.10.4 Mitochondrial transport: regulation

For initiation of axonal transport mitochondrial proteins, synaptic proteins and other membrane bound structures have to pass through the Golgi apparatus in order to receive their clathrin coat, that addresses them ready for transport (Rothman & Wieland, 1996; Stenoien & Brady, 1999). Transport over long distances is guided along microtubules, which stretch from the soma (negatively charged end) all the way to the periphery (positively charged end) (Black & Baas, 1989; Hollenbeck & Saxton, 2005; Morris & Hollenbeck, 1993). Transport within the soma and dendrites is directed mostly along actin filaments (Morris & Hollenbeck, 1995). The exact mechanisms and regulation behind the initiation of mitochondrial transport are largely unknown. However there is accumulating evidence that axon activity can influence mitochondrial trafficking.

Ca^{2+} release and uptake is certainly the best-described stop-and-go signal for mitochondria. Axonal Ca^{2+} levels vary depending on activity, branching to postsynaptic targets, mitochondrial content, external influences such as inflammation and myelination (Cai & Sheng, 2009; Ghosh-Roy, Wu, Goncharov, Jin, & Chisholm, 2010). Since Ca^{2+} is released as a response to the arrival of an action potential at the presynaptic terminal, mitochondria are needed to buffer intracellular Ca^{2+} accumulations as well as to provide ATP for Ca^{2+} pumps (Cai & Sheng, 2009). The Ca^{2+} concentration influences mitochondrial mobility through several different mechanisms, all of which are mediated by Miro, which senses Ca^{2+} with the EF hand motif and induces an uncoupling of the motor complex from the microtubule (Macaskill et al., 2009). There are several propositions of which conformational change causes this uncoupling process. Wang and Schwarz propose that Miro's Ca^{2+}-binding creates a conformational change in the kinesin complex, which detaches the motor terminal from the microtubules and connects it with Miro (Cai & Sheng, 2009; Wang & Schwarz, 2009). MacAskill suggested a slightly different mechanism, by which Ca^{2+} binds to the EF hand of Miro, which in turn distaches from KIF5, therefore disconnecting the anterograde transport unit (Macaskill et al., 2009; Rintoul, Bennett,

Papaconstandinou, & Reynolds, 2006; Wang & Schwarz, 2009). Likely this difference is due to a focus in each project. Macaskill has examined dendrites and Schwarz and Wang looked at axons (Y. Chen & Sheng, 2013). The result remains the same: Owing to this pathway mitochondria can be recruited to active synapses (Cai & Sheng, 2009). In the same experiment glutamate signaling via *N*-Methyl-D-Aspartate (NMDA) receptors has also been shown to initiate a slowing of mitochondrial transport, however this was mediated through Ca^{2+} release as well and therefore similar pathways as described above (Macaskill et al., 2009). The Ca^{2+} concentration cannot only influence the mobility and location of mitochondria, it also functions as a regulator for several different enzymes for the respiratory chain and glucose metabolism pathways such as FAD-glycerol phosphate dehydrogenase, pyruvate dehydrogenase phosphatase and even ATP synthase (Boerries et al., 2007; Tarasov, Griffiths, & Rutter, 2012). Thereby the neuron is able to steer mitochondria to a destination with low ATP concentration and at the same time upregulate ATP production through Ca^{2+} levels (Tarasov et al., 2012).

Morris and Hollenbeck could show mitochondrial accumulation upon nerve growth factor (NGF) release. This mediation was interrupted by inhibition of the phosphoinositide 3 (PI-3) Kinase pathway (Chada & Hollenbeck, 2004). This might add explanation, why mitochondria accumulate in newly forming growth cones (Morris & Hollenbeck, 1993). Furthermore, serotonin receptor 5-HT1A activation could accelerate transport towards a certain target area through AKT-glycogen synthase kinase 3β (GSK3β) pathway signaling, whereas D2 dopamine receptor antagonists and NO have been shown to inhibit mitochondrial transport (Chada & Hollenbeck, 2004; S. Chen, Owens, Crossin, & Edelman, 2007; Rintoul et al., 2006; Zanelli, Trimmer, & Solenski, 2006).

Mitochondrial morphology itself could also contribute to transport velocity and direction. As Misgeld and Kerschensteiner described, the mobile fraction was significantly shorter than the stationary fraction of axonal mitochondria, which leads to the assumption that fusion and fission processes might be connected to transport regulation (Misgeld, Kerschensteiner, et al., 2007). One explanation could be that longer tubular mitochondria are simply less flexible and

therefore harder to be carried away by the transport machinery than shorter ones (Cai & Sheng, 2009). However there exist molecular connections between mitochondrial transport and fusion and fission. Miro has been suggested to not only regulate transport via Ca^{2+}-sensing, but also to increase mitochondrial fission, whereas dynamin-related protein 1 (Drp1), a protein identified in Drosophila, responsible for fission could influence transport and mitochondrial targeting of the synapses as well (Saotome et al., 2008; Verstreken et al., 2005). Interestingly Drp1 also contains a GTPase domain like Miro (Cai & Sheng, 2009).

Since ATP has a low diffusion capacity, mitochondria must be nearby to provide replenishment (Hubley, Locke, & Moerland, 1996). In areas with a high energy demand, which might coincide with a Ca^{2+}-imbalance, mitochondrial transport is interrupted by docking to a microtubule-based protein such as syntaphylin or to actin via myosin (Chada & Hollenbeck, 2004; Kang et al., 2008). The transport velocity increases in areas high in ATP, whereas in areas low in ATP and high in ADP, such as synapses and nodes of Ranvier, it decreases (Mironov, 2007). Consequently, ATP depletion by glutamate and high ADP concentrations were able to recruit mitochondria to the stationary pool (Mironov, 2007). This might be regulated through the AMP-activated protein kinase (AMPK), which is activated with elevated energy demand, increases the mitochondrial flow into branches and outgrowing dendrites (Tao, Matsuki, & Koyama, 2014). Syntaphilin expression was also associated with branching, whereas depletion showed a reduction (M.-Y. Lin & Sheng, 2015). This suggests a link between the two processes.

Finally also extracellular glucose levels can influence mitochondrial location. The activated enzyme O-GlcNAc transferase (OGT) can lower mitochondrial motility. Its activation depends upon elevated extracellular glucose levels and induces an *O*-GlcN acetylation of Milton, which arrests mitochondrial movement (M.-Y. Lin & Sheng, 2015; Pekkurnaz, Trinidad, Wang, Kong, & Schwarz, 2014).

How do they stay on site once they arrive at their destination though? Certain regions have a complex docking apparatus in place in the presynaptic terminal called the mitochondria

associated adherens complex (MAC), first identified in Calyx of Held synapses (Rowland, Irby, & Spirou, 2000). In other places mitochondria associate with microtubular structures and neurofilaments. Especially actin filaments seem to initiate mitochondrial docking, since it has been shown that actin concentration is high in places with an accumulation of mitochondria and in places missing actin mitochondrial mobility is elevated (Hollenbeck & Saxton, 2005; Morris & Hollenbeck, 1995).

One anchor that is well described is the axonal outer-membrane protein syntaphilin. Chen and Sheng proposed the following docking mechanism. The mitochondrion moves anterogradely along the microtubule driven by kinesin. Once it passes an area of high Ca^{2+} concentration, Miro acts as a sensor and uncouples from the C-terminal of KIF5, which leaves it vacant to bind to syntaphilin, which in turn inhibits the ATPase of the motor unit (Y. Chen & Sheng, 2013). Upon deletion axonal mitochondria were more mobile and axons contained less mitochondria, whereas overexpression resulted in transport deficits (Y. Chen & Sheng, 2013). Syntaphilin could also partly explain the discrepancy in Ca^{2+}-mediated docking mechanism explained above. It is limited to axons, dendrites have different docking mechanisms possibly regulated by glutamate induced Ca^{2+} elevation resulting in a decoupling of KIF5 and mitochondria (Y. Chen & Sheng, 2013; Macaskill et al., 2009; Wang & Schwarz, 2009). If Miro should represent the primary sensor and in a neuron-specific Miro1 knock-out mouse model did not inhibit Ca^{2+} mediated anchoring, Miro2 would have to mediate this process (M.-Y. Lin & Sheng, 2015; Nguyen et al., 2014). This however has not been confirmed yet. Interestingly, syntaphilin influences anterograde and retrograde transport, although no association with dynein/dynactin has been shown yet. Possibly this is due to an inhibition of binding to dynein by a constant connection to KIF5 (Y. Chen & Sheng, 2013).

The last question is, what directs mitochondria away from the synapse and back towards the soma? Once the Ca^{2+} concentration returns to resting levels Chen and Sheng suggest that Miro once again acts as a sensor, reattaches KIF5 to the microtubules and disconnects the binding to syntaphilin (M.-Y. Lin & Sheng, 2015). They hypothesize that this is facilitated through the

continuous binding of the respective motor proteins dynein and kinesin (M.-Y. Lin & Sheng, 2015).

What happens to dysfunctional mitochondria, which ceased to produce sufficient ATP and are a source of toxic ROS? The great majority of the degradation machinery is located in the soma, the dysfunctional organelle has to be sufficiently detected and transported back. In this case the charge of the mitochondrion might play a role. Miller and Sheetz have noticed that anterogradely moving mitochondria have a higher membrane potential compared to retrogradely moving ones (Cai, Zakaria, Simone, & Sheng, 2012; K. E. Miller & Sheetz, 2004). In that way dysfunctional mitochondria get transported back to the soma and healthy ones are replenishing the distal demand (D. T. W. Chang & Reynolds, 2006). NGF earlier mentioned for its arresting potential for mitochondria has also been ascribed a role in locally modifying the their membrane potential, thereby possibly regulating their transport direction and speed (Cai & Sheng, 2009; Chada & Hollenbeck, 2004). When the membrane potential was decreased Pten-induced kinase 1 (PINK1), which was otherwise constitutively translocated from the cytosol to the mitochondrial inner membrane, cannot pass the membrane anymore, which lead to an accumulation on the outer membrane. PINK1 accumulations lead to Parkin recruitment, which in turn was shown to induce mitophagy (Ashrafi, Schlehe, LaVoie, & Schwarz, 2014). This data however stems from non-neuronal cells. In how far this mechanism applies in neurons is debatable. Cai et al. report that Parkin-targeted mitochondria are mainly found in the soma and dendrites, which is where the lysosomal degradative machinery is located as well (Cai et al., 2012). PINK mediated parkin recruitment leads to Miro phosphorylation and thereby transport arrest (Wang et al., 2011). This interaction of Parkin and PINK1 with Miro could lead to a promotion of retrograde transport or total arrest and local mitophagy instead of transport back to the soma (Ashrafi et al., 2014; S. Liu et al., 2012). It is still debated in how far local mitophagy is employed for mitochondrial quality control as opposed to retrograde transport (M.-Y. Lin & Sheng, 2015).

1.10.5 Fission and Fusion

There are various ways for an axon to remove misfolded, damaged or dysfunctional mitochondrial components. Misfolded proteins can be degraded, a dysfunctional part can be split off through fission, damaged components can be replaced through fusion with a healthy mitochondrion, or the entire mitochondrion is designated for mitophagy (H. Chen & Chan, 2009; Detmer & Chan, 2007; Westermann, 2010). Proteins responsible for mitochondrial outer membrane fusion are mitofusins 1 and 2 (MFN1, MFN2), while the optic atrophy protein 1 (OPA1) regulates the inner membrane fusion (H. Chen et al., 2003; Cipolat, Martins de Brito, Dal Zilio, & Scorrano, 2004). All three proteins are part of the dynamin family and have a GTPase domain, a middle domain, a variable domain with a transmembrane segment and a GTPase effector (GED) or coiled-coil domain (Praefcke & McMahon, 2004). In MFN the GTPase and the GED point towards the cytosol, while the transmembrane region locks the protein into the outer membrane of the mitochondria. OPA1 has a cardiolipin-binding domain instead of the variable domain (Meglei & McQuibban, 2009). The exact mechanisms of how fusion works are not known yet. It has been suggested that opposing MFNs are required that interlock and pull the membranes together (Bliek, Shen, & Kawajiri, 2013; Koshiba et al., 2004). Other possibilities would be that fusion dynamins form a spiral, in which membrane protrusions can form or forced contact between convex lipid surfaces could act fusogenic (Bliek et al., 2013). Meeusen and colleagues found out that for outer membrane fusion only GTP is needed, whereas for inner membrane fusion membrane potential is essential (Meeusen et al., 2006). Regulators for fusion proteins are ubiquitination for outer membrane fusion and proteolysis for inner membrane fusion. Ubiquitination of mitofusins can be induced by stress, once the membrane potential is decreased PINK1 and Parkin mark mitofusins for degradation (Bliek et al., 2013). However OPA1-cleaving by the metalloproteinase OMA1 and thereby preventing of inner membrane fusion is even faster than the PINK1/parkin pathway (Griparic, Kanazawa, & Bliek, 2007).

For control of fission, Drp1 and adaptor proteins are essential (Kubli & Gustafsson, 2012; Smirnova, Griparic, Shurland, & van der Bliek, 2001; Y. Yoon, Krueger, Oswald, & McNiven,

2003). As the name predicts, Drp1 is another member of the dynamin family. It has a similar structure, the variable domain most likely serves as a detection sequence for mitochondria (Bliek et al., 2013). Drp1s form a spiral cluster, which activates GTP hydrolysis, which leads to constriction of the multimer (Mears et al., 2011). Both membranes are separated during this constriction process (Bliek et al., 2013). Other than fusion, fission requires adaptor proteins for initiation. Fission protein 1 (Fis1), mitochondrial fission factor (Mff), mitochondrial dynamics proteins (MiD49, MiD51), mitochondrial elongation factor (MIEF), and the gangliosid-induced differentiation associated protein (GDAP1) have been proposed. Fis1 is important for mitochondrial fission in yeast, its role in eukaryote fission has been contested (Palmer et al., 2011). MIEF1 likely promotes fusion more than fission (Zhao et al., 2011). On balance Fis1, Mff, MiD49 and 51 possibly independently regulate fission with partially redundant functions (Bliek et al., 2013; Losón, Song, Chen, & Chan, 2013). The fission proteins and adaptors are mainly regulated through phosphorylation and ubiquitination but also other modifications like sumoylation and nitrosylation have been discussed (Bliek et al., 2013; Braschi, Zunino, & McBride, 2009; Cho et al., 2009). Interactions with the cytoskeleton and the endoplasmic reticulum have been described as well (Bliek et al., 2013).

Twig et al. have described a circle of events, starting with a single mitochondrion, which fuses with other mitochondria to form a network, from this network dysfunctional parts can be discarded by fission. The membrane potential of discarded pieces is usually lower and can thus be targeted for mitophagy. However, recovery of the daughter mitochondria can lead to reuptake into the network and formation of single unit mitochondria (Twig et al., 2008). Van Bliek interpreted increased fusion and decreased fission as favorable for cell survival (Bliek et al., 2013).

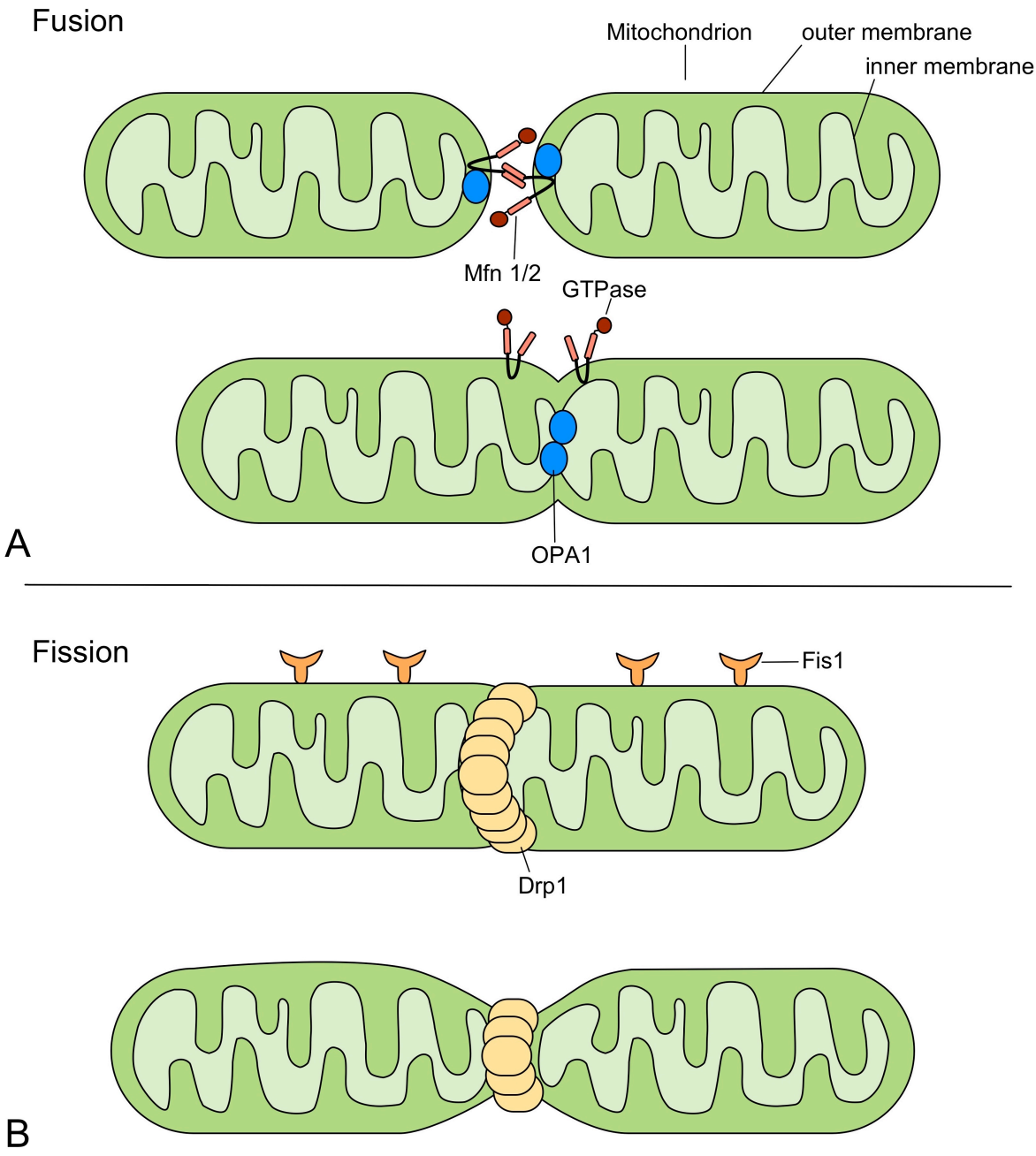


Fig. 1-7 Fission and Fusion

In order for two mitochondria to fuse a close alignment is needed. Mfn1 and 2 are responsible for outer membrane fusion and OPA1 is located on the inner membrane and initiates inner membrane fusion. Fission is initiated by adaptor proteins such as Fis1, which recruit Drp1 to the surface of the mitochondrion. Drp1 form a multimeric spiral complex, which constricts the membrane and completes fission. Adapted from Chen and Chang 2006 (H. Chen & Chan, 2006). Rights were obtained from Elsevier.

1.10.6 Mitochondrial pathology in MS – chicken or egg

Mitochondrial pathology in MS has been the focus of many groups for the past decades. In how far mitochondrial pathology is the cause or the consequence is still subject of debate. Mitochondrial DNA-defects and deficient DNA repair, abnormal mitochondrial gene expression patterns, and dysfunctional or defective mitochondrial enzymes, increased free radicals and oxidative damage, and fission and fusion imbalance can lead to mitochondrial dysfunction (Mao & Reddy, 2010). Judging by its indispensable function for the neuron it is easy to imagine how mitochondrial dysfunction can lead to neurodegeneration. In many neurodegenerative diseases a link with mitochondrial dysfunction has already been established. Familial forms of Parkinson's disease, Alzheimer's disease, Amyotrophic lateral sclerosis, Huntington's disease, Friedreich ataxia and hereditary spastic paraplegia have been reported to have underlying mitochondrial pathology (Schon & Manfredi, 2003).

Only few mtDNA and nuclear DNA alterations have been described in conjunction with MS. Age-related variations in mitochondrial DNA, the single nucleotide polymorphism nt13708 G/A, an over-representation of super-haplogroup U and carriers of haplogroup JT, changes in the nuclear encoded complex I gene NDUFS2 and the uncoupling protein 2 (UCP2) promoter polymorphism 866G/A might influence the susceptibility for MS (Ban et al., 2008; Mao & Reddy, 2010; Vogler et al., 2005; Yu et al., 2008).

The genetic component influencing and impairing mitochondrial function appears to be relatively small but might still be very relevant in some familial cases. This could however not explain the striking lack of mitochondrial content in the spinal cord of MS patients and reported widespread mitochondrial dysfunction in MS. Lack of sufficient energy supply as a result of demyelination, Ca^{2+} influx and general metabolic imbalance is the major cause for axonal degeneration. A gene expression pattern analysis revealed reduced neuronal mitochondrial gene expression and reduced respiratory chain complex I and III functional activity (Dutta et al., 2006). Different stages of de- and remyelination in active and chronic lesions have been found to have different mitochondrial content. In active lesions a decreased mitochondrial content has been found, which is most likely due to edema and tissue loss (D. Mahad et al., 2008). Moreover complex I and IV of the mitochondrial respiratory chain were found defective in

acute pattern III lesions (D. Mahad et al., 2008). Following demyelination an increase in mitochondrial content has been observed. Chronically demyelinated axons revealed an increased complex IV activity and increased mitochondrial mass (Andrews et al., 2006; D. J. Mahad et al., 2009; Ohno et al., 2014). Axonal syntaphilin was also increased in chronically demyelinated lesions (D. J. Mahad et al., 2009). The complex IV activity was inversely correlated with the inflammatory cell density in the lesion (D. J. Mahad et al., 2009). Upon remyelination, an increased content of mitochondria has been reported as well, but not as pronounced as in acute and chronically demyelinated axons (Zambonin et al., 2011). On the one hand these results speak for a significant contribution of mitochondrial changes to the pathology of MS, on the other it also shows a compensatory role of mitochondrial accumulations in areas of energy deficits. Possibly those deficits can be compensated for the duration of the relapsing remitting phase. Progression might be due to a failure of that compensation as shown in a study on CSF of MS patients, which found lactate, sorbitol and fructose more increased in SPMS patients than in RRMS patients suggesting a metabolic impairment (Regenold, Phatak, Makley, Stone, & Kling, 2008). Only recently the research group around van Horssen measured serum lactate levels in MS patients and healthy controls and found that increased lactate, as a measurement of mitochondrial dysfunction, was associated with disease severity (EDSS), upper limb motor function, walking disability, loss of color vision (Petzold et al., 2015). Additionally muscle tissue of MS patients showed less type I fibers, smaller fibers over all and lower succinate dehydrogenase levels, which is part of complex II of the respiratory chain (Kent-Braun et al., 1997; Mao & Reddy, 2010). Inactivation studies on the permeability transition pore and its regulator cyclophilin D have shown a resistance against oxidative agents and faster recovery from EAE (Forte et al. 2007).

1.12 Synapse pathology

1.12.1 Synapse regulation

Being the functional unit of the neuron, synaptic integrity is indispensable for neuronal transmission. Synapses in the CNS are constantly being abandoned and reformed, depending on their usage and the environment. During neurodegenerative diseases like Huntington's and Alzheimer's disease, synaptic loss is often extensive and has been described to positively correlate with clinical disease (Jebelli, Su, Hopkins, Pocock, & Garden, 2015).

Different glial cell populations influence synapse number and integrity in different ways.

First, astrocytes have been shown to aid in neuronal circuit formation in the developing and adult brain, especially excitatory glutamatergic synapses (Jebelli et al., 2015). They can influence synapse function directly by modulating glutamate and Ca^{2+} signaling, or they engulf synaptic material and thereby eliminate excessive synapses (Chung et al., 2013; Jebelli et al., 2015; Newman, 2003). In ALS astrocyte dysfunction has been reported to lead to glutamate excitotoxicity, which can provoke neuronal damage (Jebelli et al., 2015; Nagai et al., 2007). Elevated glutamate concentrations have been found in CSF from MS patients to correlate with disease severity, and glutamate excitotoxicity has also been described as a pathogenetic factor in mitochondrial and axonal damage in MS (Barkhatova, Zavalishin, Askarova, Shavratskii, & Demina, 1998; Pitt, Werner, & Raine, 2000; Srinivasan, Sailasuta, Hurd, Nelson, & Pelletier, 2005; Stover, Lowitzsch, & Kempski, 1997; Zhu, Luo, Moore, Paty, & Cynader, 2003). Furthermore, astrocyte glutamate uptake is inhibited by ROS and RNS released by microglia and macrophages in EAE and MS (Piani, Frei, Pfister, & Fontana, 1993).

Secondly, microglia as well have an important role in synapse elimination and surveillance. Wake and colleagues demonstrated how microglial processes constantly survey synapses by contacting them for approximately five minutes each hour. Interestingly, microglial motility is dependent on neuronal function. With reduced neuronal activity, microglial dendrite

movement slows down (Wake, Moorhouse, Jinno, Kohsaka, & Nabekura, 2009). Briefly, during normal brain function in the resting state, microglia can mediate synaptic plasticity via TNFα release, shape neuronal circuits by phagocytosing synaptic structures, and promote synaptic growth during development by releasing growth factors (Jebelli et al., 2015; Paolicelli et al., 2011; Stellwagen & Malenka, 2006; Tremblay et al., 2011). Upon activation by hypoxia, lipopolysaccharide (LPS) or other inflammatory stimuli microglia retract their processes, which primarily leads to a decreased surveillance function and secondarily to loss of inhibitory synapses, promotion of excitatory synapses, increased long-term-synaptic depression, and neuroprotective signaling (Z. Chen et al., 2014; Jebelli et al., 2015; Zhang et al., 2010).

During neurodegenerative diseases and aging, microglia become dysfunctional and then play a major part in synapse loss in neurodegenerative disease, which is said to occur simultaneously with inflammation and clinical decline (Bessis, Béchade, Bernard, & Roumier, 2007).

Lastly, myelin-forming cells have been shown to influence synaptic presence in the CNS. Especially oligodendrocyte precursors proliferate upon demyelination and inhibit synaptic activity when dysfunctional (Kukley, Nishiyama, & Dietrich, 2010; Sahel et al., 2015). Schwann cells in the peripheral nervous system can sense neuronal activity and modulate transmission (Feng, Koirala, & Ko, 2005; Jebelli et al., 2015).

All those cells can influence synapses from the outside. The activity of the neuron and this particular neuronal connection is an important intrinsic factor for synapse stability. It is likely that mitochondrial content is a determining factor for synapse integrity. It is possible that with a downregulation of supply of mitochondria for the synapse i.e. during acute transport block proximal along the axon the synapses would lack mitochondria as well.

2 Important work prior to mine

2.1 Focal axonal degeneration

Ivana Nikić and colleagues described a new variant of axon loss in inflammatory EAE lesions. First, she distinguished between three sequential stages and termed the entire process 'focal axonal degeneration' (FAD). During FAD the axons form focal swellings within areas of inflammatory cell infiltration (stage 1), and eventually start to fragment (stage 2). Using confocal microscopy they observed mitochondrial changes that occur in normal-appearing axons before any alterations of the axonal morphology could be observed (stage 0b). The normal appearing axon with normal appearing mitochondria is defined as stage 0a. Mitochondrial changes were measured by means of the shape factor (length divided by width). A rounded shape, which coincided with a loss of internal structure, confirmed by electron microscopy, characterized damaged mitochondria (Nikić et al., 2011).

After having determined the individual stages, they focused on describing how these morphological changes developed over time and space and discovered the following key characteristics: First, they discovered that FAD is mostly restricted to the lesion area and thus closely connected to the presence of immune cells including activated macrophages/ microglia. It is a reversible process that can recover spontaneously over time and recovery can be further facilitated by the addition of ROS scavengers. Secondly, ultrastructural analysis revealed that axonal and mitochondrial pathology can occur independently of demyelination. Thirdly, mitochondrial pathology seems to be an important contributing factor to axon degeneration in EAE. Treating healthy mice with an uncoupling agent targeting the respiratory chain of mitochondria caused mitochondria breakdown soon followed by FAD-like axon degeneration. Finally they were able to find correlates of FAD in biopsy tissue from MS patients. The human tissue samples showed, similar to EAE, many axons with intact myelin sheaths that displayed FAD-like axonal and mitochondrial changes. Targeting mitochondria seemed to be a promising new approach for treating MS. This required more research on mitochondrial function and properties (Nikić et al., 2011).

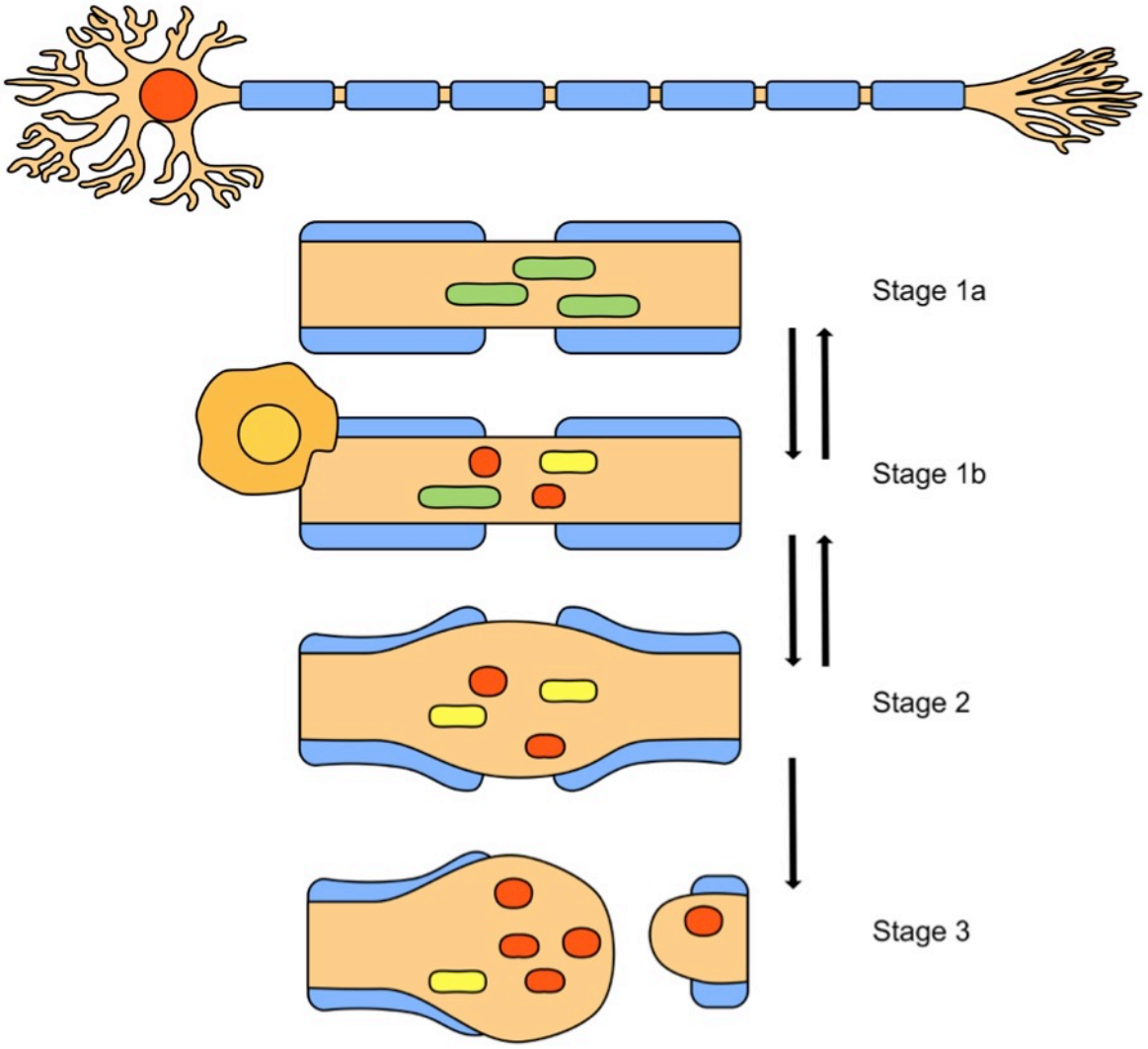


Fig. 2-1 Focal axonal degeneration in EAE adapted from Nikić et al. 2011 (rights were obtained from Nature Publishing Group)

2.2 Pervasive axonal transport deficits

Mitochondria are highly dynamic cell organelles that are transported from the neuronal soma to the periphery (anterograde transport) and from the periphery back to the soma (retrograde transport). Catherine Sorbara focused her research on the transport changes of mitochondria and other cell organelles inside and outside of an EAE lesion (C. D. Sorbara et al., 2014). She used *in vivo* two-photon imaging of individual axons within the spinal cord (Davalos et al., 2008;

Nikić et al., 2011) and fluorescently labeled organelle cargoes namely mitochondria (Thy1-MitoCFP mice; and peroxisomes (in Thy1- PeroxiYFP mice) to assess this process (Misgeld, Kerschensteiner, et al., 2007).

2.2.1 Mitochondrial transport inside an acute lesion

By imaging mitochondrial transport in axons that cross acute neuroinflammatory lesions, Sorbara found lower transport rates in anterograde as well as in retrograde direction. Interestingly, those changes could be observed not only in stage 1 (=swollen) axons, but also in stage 0 (normal appearing) axons. While transport speed and stop frequency were only moderately altered, the stop duration was significantly longer in stage 0 EAE axons compared to control axons. This helps to explain the raised mitochondrial density and content in axons (stage 0 and 1) within the lesion area compared to controls (relative to control axons: 111% ± 5% of mitochondrial content in stage 0 axons, 128% ± 9% in stage 1 axons) (C. D. Sorbara et al., 2014).

Similarly, Witte and colleagues found an increased number of mitochondria in axons in MS tissue (Witte et al., 2014). From this knowledge the question arose, whether this accumulation originated from focal structural changes of the axon, cargoes or microtubule tracks. Interestingly, changes in transport preceded not only demyelination or dysmyelination but also mitochondrial changes and alterations of the microtubule tracks (Edgar et al., 2004; Kiryu-Seo, Ohno, Kidd, Komuro, & Trapp, 2010; Nikić et al., 2011; Ohno et al., 2014; Romanelli et al., 2013; Witte et al., 2014).

2.2.2 Mitochondrial transport proximal to a lesion

Dorsal root axons proximal to the lesion showed comparatively elevated retrograde and normal anterograde transport, indicating a transport stop at the site of the lesion that may cause a reversal of transport from there back to the cell body, similar to what occurs following axon transection in vitro (Misgeld, Kerschensteiner, et al., 2007; C. D. Sorbara et al., 2014).

2.2.3 Amelioration of transport deficits by anti-inflammatory drugs

In the clinical treatment of an exacerbation of MS, corticosteroids are used to mitigate the severity and accelerate the process of remission. Catherine Sorbara showed that the recovery of transport deficits after the acute peak of EAE could be accelerated by application of corticosteroids as well. Transport deficits could be initiated by application of NO donors in healthy mice in a concentration that would not affect the axonal or mitochondrial structure, and could be reversed by local application of a NO scavenger. This suggests that transport deficits are associated with an early inflammatory stage and represent a process of reversible axonal dysfunction (C. D. Sorbara et al., 2014).

2.2.4 Mitochondrial transport in chronic neuroinflammatory lesions

In a chronic EAE model those transport deficits could be shown to recover to some extent after the acute phase, but not entirely. Strikingly the anterograde transport rates appeared to be more severely affected than the retrograde transport rates. As a result the distal parts of the axons passing through a lesion received several hundreds of mitochondria less per day than healthy axons. This indicates that transport deficits could over time lead to a net deprivation of these important organelles in the axonal segments that are distal to a neuroinflammatory lesion (C. D. Sorbara et al., 2014).

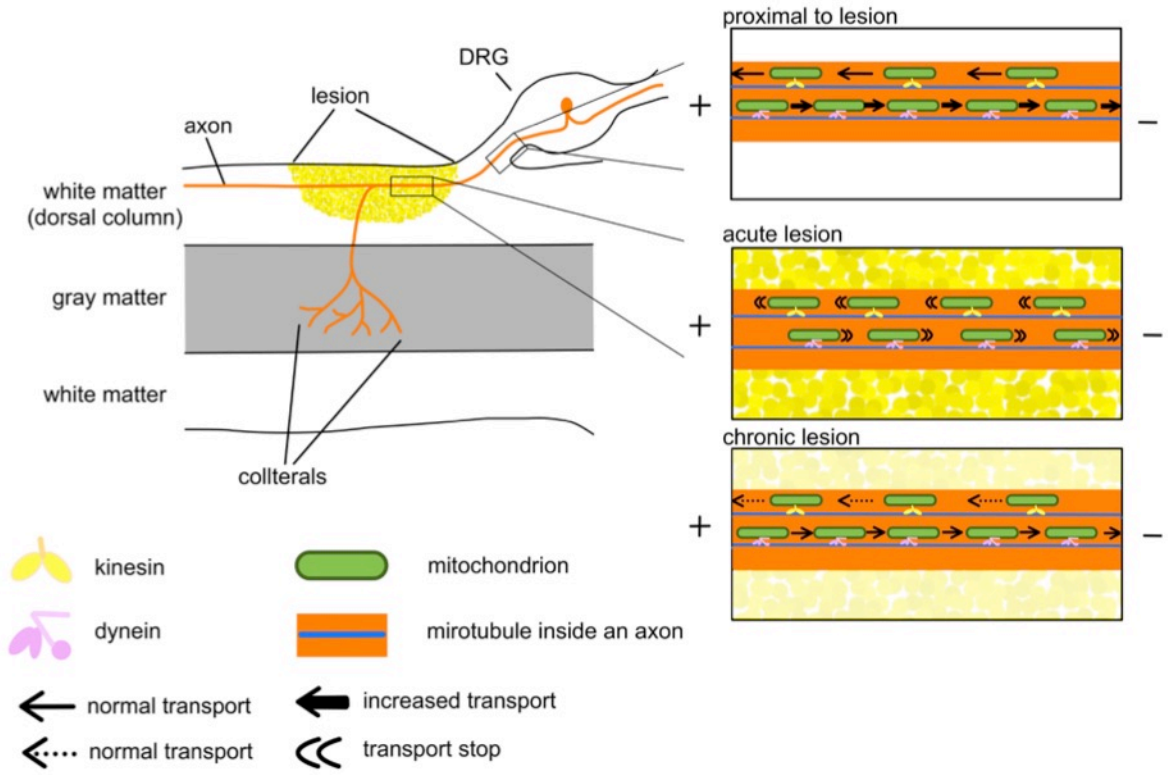


Fig. 2-2 Transport deficits in and around the lesion

Schematic of transport deficits within the lesion and proximal to it. Proximal retrograde transport is increased while anterograde transport is unaltered. Inside the acute lesion transport stops entirely. During recovery in the chronic phase the lesion reconstructs itself, inflammatory cells are spread out more diffusely and transport continues with more deficits in anterogradely (partly adapted from Sorbara et al., 2014).

3 Material and Methods

3.1 Materials

3.1.1 Reagents

Preparation of surgery	
Ketamine hydrochloride 10% (Ketamine)	Bremer Pharma GmbH, Warburg, Germany
Xylariem 20mg (Xylazine)	Riemser Arzneimittel AG, Greifswald-Insel Riems, Germany
Forene (Isoflurane)	Abbott AG, Baar, Switzerland
Bepanthen Augen- und Nasensalbe 5g (eye cream)	Bayer Vital GmbH, Leverkusen, Germany
Cutasept F Lösung 250ml (disinfectant spray)	Bode Chemie GmbH & Co, Hamburg, Germany
DRG-Injection	
rAAV-*CAG-mOrange* (monomeric Orange) titer: $9x10^{12}$ genome copies/ml, diluted 1:5 in sterile PBS	produced by Anja Schmalz (Institute of Clinical Neuroimmunology)
PBS 10x (phosphate buffered saline), pH= 7,2/7,4	1l PBS 10x (pH 7,2/7,4) 2,6g NaH_2PO_4 (M=137.99g/mol) 14,4g $Na_2HPO_4x2H_2O$ (M=177,99g/mol) 87,5g NaCl
Isotone NaCl (Sodium Chloride) 0.9% 500ml	B Braun Melsungen AG
Immunization	
myelin oligodendrocyte glycoprotein (MOG, N1-125, expressed in E.coli)	Kindly provided by Dr. Doron Merkler, University of Geneva or made by in-house technician

NaAcetate	
Complete Freund's adjuvant	Sigma-Aldrich, St.Louis (Missouri), USA
Mycobacterium tuberculosis H37Ra	Difco Laboratory, Detroit, MI, USA
Pertussis toxin	Sigma-Aldrich, St.Louis (Missouri), USA
Perfusion	
Heparin-Sodium 25000 Ratiopharm	Ratiopharm ® Ulm, Germany
Paraformaldehyde 4%	Preparation described below 8% PFA (Sigma-Aldrich) in dH2O, heated up to 55°C and stirred additional 10min, filtrated and mixed in a 1:1 ratio with 0,2M PB (Phosphate buffer), pH adjusted to 7,2- 7,8
Cyrofixation, Immunohistochemistry and Storage	
0.1%/20% NaN_3/H_2O	Merck KGaA, Darmstadt, Germany
Triton X-100 C34H62O11 Sigma S	Sigma-Aldrich, St.Louis (Missouri), USA
Sucrose	Sigma-Aldrich, St.Louis (Missouri), USA
Gibco goat serum	Invitrogen GmbH, Darmstadt, Germany
NeuroTrace ® 500/525 fluorescent Nissl stain	Invitrogen GmbH, Darmstadt, Germany
Rabbit x Anti-Synapsin I Polyclonal antibody 'AB1543	Merck Millipore (division of Merck KGaA, Darmstadt, Germany)
Synaptophysin (D35E4) XP ™ Rabbit mAB #5461	Cell Signaling Technology, Inc., Danvers, USA
Alexa-Fluor® 633 goat-anti-rabbit IgG (H+L) 2mg/ml MPA-21094	Invitrogen GmbH, Darmstadt, Germany

Alexa-Fluor® 647 donkey anti-rabbit IgG (H+L) 2mg/ml in 0.1M NaP, 0.1M NaCl, pH 7.5, 5mM azide A31573	Invitrogen GmbH, Darmstadt, Germany
Tissue Tek ® OCT ™ Compound	Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands
Vectashield Mounting Medium	Vector Laboratories, Inc., Burlingame (California), USA

3.1.2 Tools and materials

Preparation of surgery	
Support pillow	Custom-made
Metal plate	Custom-made
Cast Alnico Button Magnets (magnets)	Eclipse Magnetics Ltd, Sheffield, UK
Rubber bands	
Micro Pipettes Blaubrand ® intraMARK 1/2/3/4/5µl (micropipettes/glass capillaries)	Brand GmbH und Co. KG, Wertheim, Germany
Plastipak Syringe 20ml Luer Lok ™ (syringe for virus injection)	BD™ Becton, Dickinson (New Jersey) and Company, Franklin Lakes, USA
Syringe 2ml Injekt ® luer solo (syringe for injection of Ringer's solution)	B. Braun Melsungen AG, Melsungen, Germany
Plastipak Syringe 1ml (for Ketamine and Xylazine application)	BD™ Becton, Dickinson (New Jersey) and Company, Franklin Lakes, USA
Sterican ® Hypodermic needle 0.3x12mm BL/LB 30Gx ½" (KX injection)	B. Braun Melsungen AG, Melsungen, Germany
Hypodermic needle Microlance ™ 3 27G ¾" Nr. 20 (NaCl Injection)	BD™ Becton, Dickinson (New Jersey) and Company, Franklin Lakes, USA
Hypodermic needle Microlance ™ 3 23G ¾" Nr. 20 (hydration during	BD™ Becton, Dickinson (New Jersey) and Company, Franklin Lakes, USA

surgery)	
Safety-Multifly®-Set 21Gx ¾“ TW 0.8x19mm (blood collecting system)	SARSTEDT AG & Co., Nümbrecht, Germany
Eppendorf plastic tubes	Eppendorf
Surgical Instruments	
Feather stainless steel blade (surgical blade)	Feather Safety Razor Co LTD
Noyes Spring Scissors (titanium, 12cm, 14mm blades, tip diameter 0.275, angled up 15013-12) stainless	Fine Science Tools GmbH, Heidelberg, Germany
Vannas-Tübingen Spring Scissors, stainless 15003-08	Fine Science Tools GmbH, Heidelberg, Germany
Dumont #3 Forceps –Inox Alloy (11cm, straight, 0.17mm x 0.1mm, 11231-20)	Fine Science Tools GmbH, Heidelberg, Germany
Dumont #5 Forceps – DUMOSTAR ® Inox Alloy (11cm, straight tip, tip dimensions 0.1mm x 0.06mm, 11251-20)	Fine Science Tools GmbH, Heidelberg, Germany
Dumont #2 Laminectomy Forceps - Inox Alloy (12 cm, straight tip, 0.4mm x 0.06mm, 11223-20)	Fine Science Tools GmbH, Heidelberg, Germany
Friedman Pearson Rongeur curved, 0.5mm, 14cm16221-14	Fine Science Tools GmbH, Heidelberg, Germany
Halsey Needle Holder, Serrated Tungsten Carbide 12501-13	Fine Science Tools GmbH, Heidelberg, Germany
Sugi (absorbent triangles)	Kettenbach GmbH & Co. KG, Eschenburg, Germany
Cotton applicator	NOBA Verbandmittel Danz GmbH und Co KG
Olsen-Hegar Needle Holder	Fine Science Tools GmbH, Heidelberg, Germany
Ethicon Ethilon monofil polyamide 6-0, 667H (skin suture)	Johnson & Johnson Medical GmbH, Norderstedt, Germany

Ethicon Vicryl pooyglactin 910 4-0 BB, 20cm MIC101H (muscle suture)	Johnson & Johnson Medical GmbH, Norderstedt, Germany
Immunization	
Luer lock 1ml 1000 series GASTIGHT syringe #1001	Hamilton Bonaduz AG, Bonaduz/Switzerland
Plastipak Syringe 1ml (for Ketamine and Xylazine application)	BD™ Becton, Dickinson (New Jersey) and Company, Franklin Lakes, USA
Sterican ® Hypodermic needle 0.3x12mm BL/LB 30Gx ½“ (KX and MOG injection)	B. Braun Melsungen AG, Melsungen, Germany
Perfusion and dissection	
Cellstar tubes 50ml, PP, graduated, conical bottom, blue screw cap	Greiner Bio-One GmbH, Frickenhausen, Germany
Cellstar tubes 15ml, PP, graduated, conical bottom, blue screw cap	Greiner Bio-One GmbH, Frickenhausen, Germany
Haemostat scissors 12301-13	Fine Science Tools GmbH, Heidelberg, Germany
Dumont Forceps 1026-15	Fine Science Tools GmbH, Heidelberg, Germany
PFA preparation	
pH-test sticks 4.5-10.0 C731	Laborbedarf ROTH
Magnetic stirring bars	Heidolph Instruments GmBH & Co. KG, Schwabach, Germany
Erlenmeyer flask (1l, 2l)	Schott, Elmsford (New York), USA
Whatman® quantitative filter paper, ashless, circles Grade 41, 150cm	Whatman® Sigma-Aldrich, St.Louis (Missouri), USA
Plastic funnel	
Thermometer	

Cryofixation, Cutting, Immunohistochemistry	
Tissue Tek Cryomold Biopsy, Disposable Vinyl Specimen Molds, 15x15x5mm, 10x10x5mm	Sakura Finetek Europe B.V. , Alphen aan den Rijn, The Netherlands
VWR ® Petri Dishes, sterile	VWR International, Radnor, USA
Glass bottles 500ml	Schott Duran, Elmsford (New York), USA
Cellstar tubes 15ml, PP, graduated, conical bottom, blue screw cap	Greiner Bio-One GmbH, Frickenhausen, Germany
Eppendorf tubes (1.5ml, 2ml)	Eppendorf AG, Hamburg, Germany
Pipettes and pipette tips (2-20µm, 20-200µm, 100-1000µm)	Eppendorf AG, Hamburg, Germany
FALCON ® Non-tissue culture treated plates, flat bottom with low evaporation lid (6- wells, 12-wells, 24-wells)	FALCON ® VWR International, Radnor, USA
FALCON ® Cell culture Inserts 3.0µm pore size PET track-etched membrane	FALCON ® VWR International, Radnor, USA
Microscopic slides 76x26mm Thermo Scientific ground edges 90° frosted end	Thermo Fisher Scientific Inc,, Waltham, Massachusetts, USA
Microscope cover slips 24x50mm, 24x32mm	Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany
Laboratory Film, Parafilm PM966	Pechiney Plastic Packaging, Inc, Chicago, USA

3.1.3 Technical devices, Software

Preparation for surgery	
Wella contura HS61 (Hair clipper)	Wella, Darmstadt, Germany
Heat therapy pump Model #TP702 (for heating pad for anaesthetized	Gaymar Industries, Orchard Park (New York), USA

mice)	
Grundig Microban little Guard (vacuum cleaner for collecting hair while shaving mice)	Grundig, Nürnberg, Germany
Vertical Micropipette Puller Model P-30	Sutter Instrument Company, Novato (California), USA
Vortex-Genie 2	Scientific Industries, Inc., Bohemia (New York), USA
Electrode Manipulator, small animal microinjection system	David Kopf Instruments, Tujunga (California), USA
Technical devices for surgery, immunohistochemistry, PFA preparation	
Olympus Stereo Microscope SZ51 (zoom range 0.8-4)	Olympus GmbH, Hamburg, Germany
Olympus KL 1500 LCD (cold light system for stereo microscopy)	Olympus GmbH, Hamburg, Germany
Olympus SZX16 fluorescence stereomicroscope (zoom range 0.8-4, Dissection microscope)	Olympus GmbH, Hamburg, Germany
X-Cite Series 120 Fluorescence Microscope Light Source	Excelitas Technologies Corp. U.S.A.
Magnetic stirring hotplate MR 3001K	Heidolph Instruments GmBH & Co. KG, Schwabach, Germany
Leica CM1850 cryostat	Leica Microsystems GmbH, Wetzlar, Germany
Ismatec IP High Precision Multichannel Pump	IDEX Health & Science GmbH, Futtererstr. 16, 97877 Wertheim, Germany
AND EK-2000i Digital Lab Scale	A&D Company, Limited, Tokyo, Japan
IKA vibrax VXR (shaker)	IKA®-Werke GmbH & CO. KG, Staufen, Germany
Olympus IX71 inverted fluorescence microscope	Olympus GmbH, Hamburg, Germany

	Confocal microscope	Olympus GmbH, Hamburg, Germany
	ImageJ/ FIJI	National Institutes of Health, Bethesda, USA
	Fluoview FV1000	Olympus GmbH, Hamburg, Germany
	Microsoft Office 2011	Microsoft Inc., Redmond, USA

3.1.4 Subjects

Transgenic animals

To study acute EAE the following mice lines were used: *Thy1-MitoCFP-S, Thy1-MitoCFP-P* mice (Jackson Laboratory strain designation: Tg(Thy1-CFP/COX8A)S2Lich/J) mice)

To study chronic EAE the following mice lines were used: Biozzi ABH x *Thy1-MitoCFP-S* and Biozzi ABH x *Thy1-MitoCFP-P*. BiozziABH mice were obtained from Harlan Laboratories (strain designation BiozziABH/RijHsd) and were crossed with *Thy1-MitoCFP*-S or *Thy1-MitoCFP-P* mice. F1 mice were analyzed. All mice were between 6-12 weeks old when experiments began (C. D. Sorbara et al., 2014).

Transgenic mouse lines express a mitochondrially targeted CFP, cyan fluorescent protein, under the control of the neuron-specific thy1-promotor (Misgeld, Kerschensteiner, et al., 2007). They weighed between 20g and 30g at the beginning of the experiments. The animals were housed in groups of two to five and given food and water *ad libitum*. In addition, they were provided with nesting material and possibilities to retreat.

All experiments using mice were performed in accordance with the institutional guidelines and approved by the Study Committee of the Regierung von Oberbayern.

3.2 Methods

3.2.1 Induction of Acute Experimental Autoimmune Encephalomyelitis

Induction of EAE was carried out by Catherine Sorbara according to a standard protocol (Abdul-Majida et al., 2000). Here is a brief overview of the procedure.

The solution for immunization consisted of a 1:1 mixture of 200-350µg purified recombinant myelin oligodendrocyte glycoprotein (MOG, N1-125, expressed in E.coli, was kindly provided by Dr. Doron Merkler, University of Geneva or made by in-house technician), diluted down to the desired concentration (µg/µl) with sodium acetate and complete Freund's adjuvant (Sigma) with 5 mg/ml mycobacterium tuberculosis H37 Ra (Difco). This solution was mixed with two Hamilton syringes with a connecting duct until completely emulsified and then injected subcutaneously on each side (2x100µl) and on the base of the tail (50µl). Pertussis toxin was administered intraperitoneally in dosages of 250-400ng on day 0 and day 2 following immunization (C. D. Sorbara et al., 2014).

3.2.2 Induction of Chronic Experimental Autoimmune Encephalomyelitis

A chronic relapsing form of EAE was induced as follows. In this case mice were injected subcutaneously with emulsion twice, on day 0 and 7. The solution contained 100-200µg purified recombinant MOG (N1-125). On day 0 and day 1 following each immunization 100-200ng Pertussis toxin was injected intraperitoneally (Baker et al., 1990; C. D. Sorbara et al., 2014).

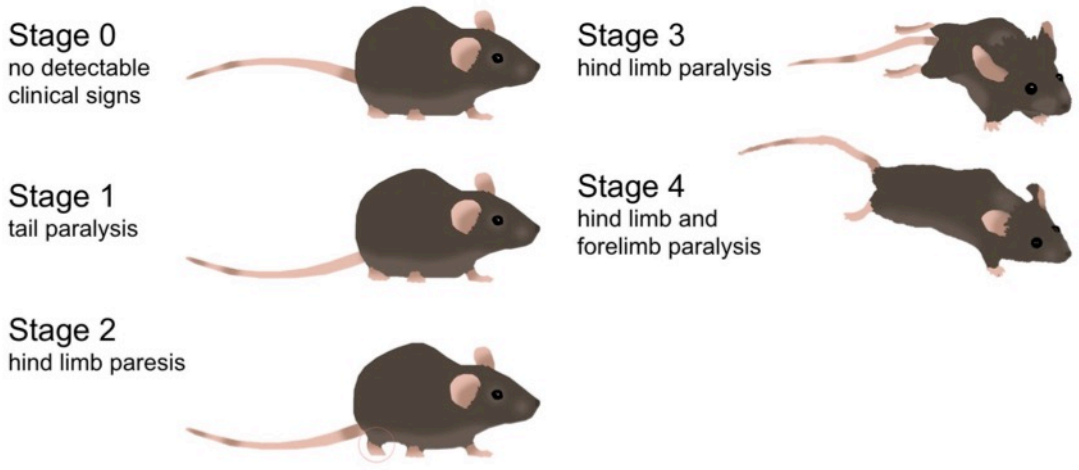
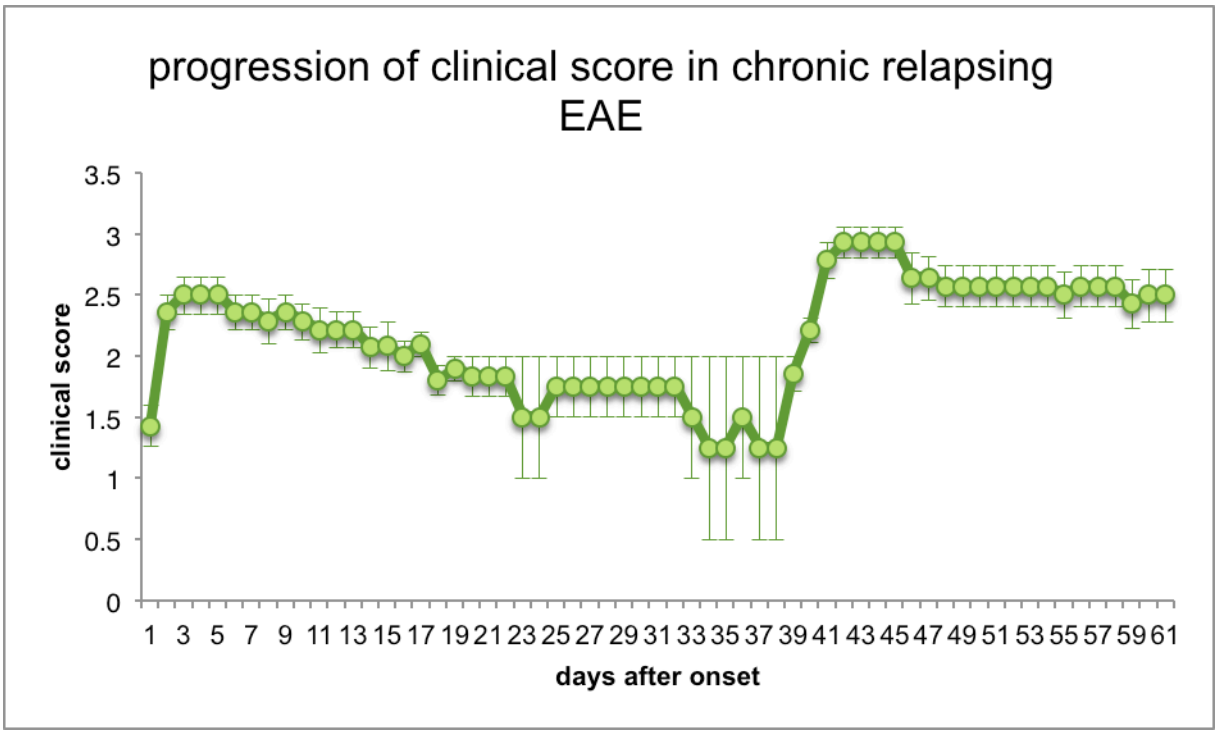


Fig. 3-1 Progression of clinical score in EAE

The mice are placed on a plane surface and observed for their spontaneous movement. Motor function of tail, hind limbs and fore limbs is evaluated on the displayed scale.



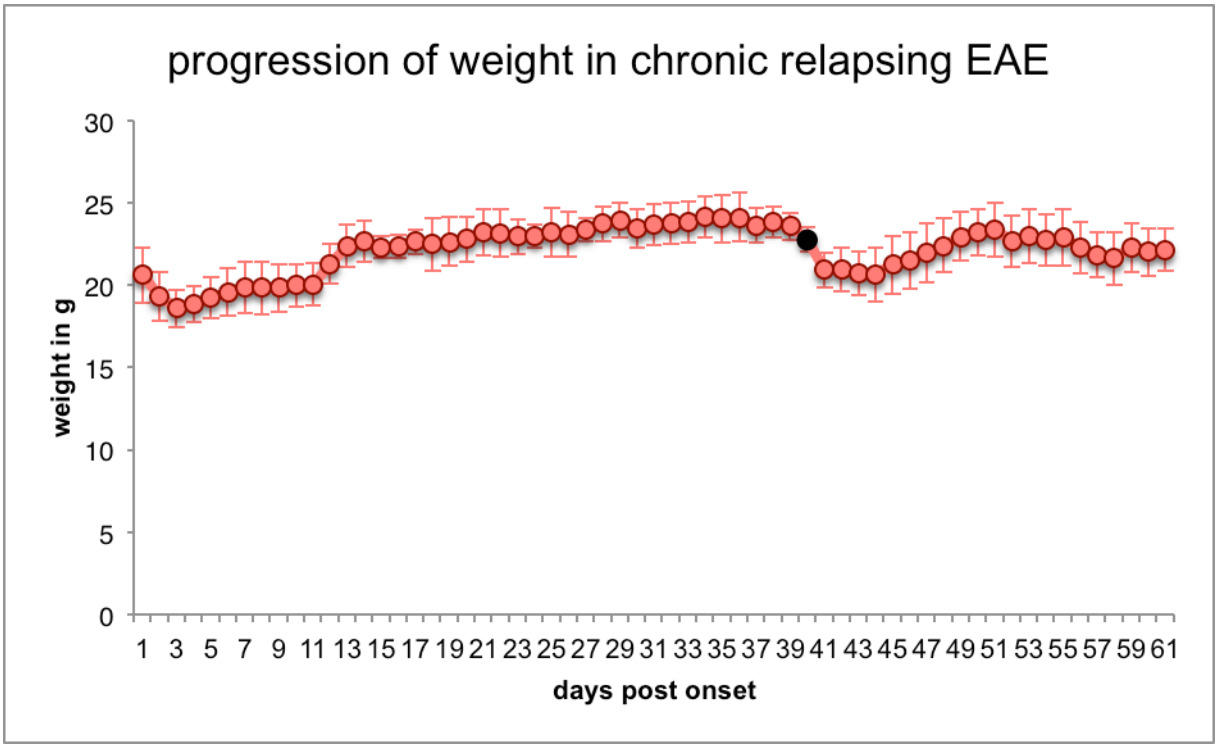


Fig. 3-2 Progression of clinical score and weight in the chronic relapsing form of EAE
This representative disease course is calculated from 7 Biozzi ABH x *Thy1-MitoCFP-S* mice. The black data point indicates the average day of relapse after remission (on average 40 days after onset of disease and around 50 days after immunization). Each point represents mean ± s.e.m..

3.2.3 Clinical Scoring

The animals were given a clinical score depending on their level of disability and weighed daily. The standard scoring system for EAE, described by Abdul-Majid and used in our paper by Sorbara as well, was used with the following parameters: 0, no detectable clinical signs; 0.5, partial tail weakness; 1, tail paralysis; 1.5, gait instability or impaired righting ability; 2, hind limb paresis; 2.5, hind limb paresis with partial dragging; 3, hind limb paralysis; 3.5, hind limb paralysis and forelimb paresis; 4, hind limb and forelimb paralysis; 5, death (Abdul-Majid et al., 2000; C. D. Sorbara et al., 2014). This information was plotted on a graph and used to determine the time point of injection and perfusion (Fig. 3-2).

3.2.4 Surgery – Dorsal Root Ganglion (DRG) Injection

Preparation

Glass pipettes were prepared with a solution of rAAV-*CAG-mOrange* (titer: $9x10^{12}$ genome copies/ml, diluted 1:5 in sterile PBS), made visible by adding diluted methylene blue. The mice were anesthetized via intraperitoneal injection with a solution made out of Ketamine and Xylazine (ketamine 87µg per g body weight, xylazine 13µg per g body weight). Half the dosage of anesthesia was re-administered once per hour for the duration of the surgery. The level of anesthesia was tested with the toe pinch reflex.

An electric clipper was used to shave the hair in the area of surgery. Remaining loose hair was removed with a damp swab. The mouse was positioned on a custom made plate with a padding. The extremities were fixated on this construction with elastic straps connected to adjustable magnets. A supporting pillow was placed underneath the abdomen of the mouse in order to widen the inter-laminar spaces. To prevent dryness and irritation of the eyes, an ointment was applied (Bepanthen® Augen- und Nasensalbe).

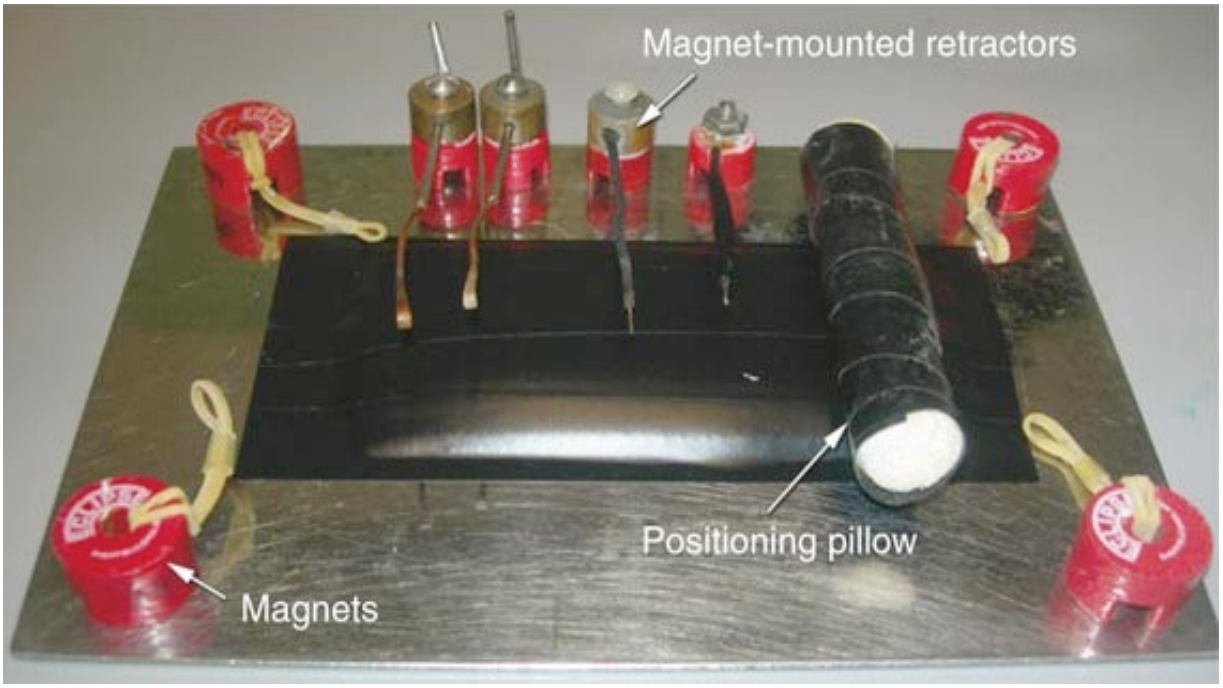


Fig. 3-3 Equipment for animal positioning
The mouse is fixed upon the stage with magnet-mounted rubber bands. The positioning pillow is placed underneath the lower abdomen, in order to allow a widening of inter-laminar spaces to facilitate the laminectomy. Skin and muscles are pulled to the sides with customized magnet-mounted retractors to give a clear view of the surgical site. Image adapted from (Misgeld, Nikic, & Kerschensteiner, 2007).

Laminectomy

This procedure was performed under a surgery stereomicroscope at 3-4-fold magnification. After disinfection of the shaved skin area with Cutasept, a 20mm long skin incision was made over the laminectomy site extending from the last thoracic vertebra Th12 to the sacrum. The lumbar vertebra L5 was located and an incision was made to both sides of the spinal protrusions. The muscles were scraped away, careful to not break off any bone fragments or harm the spinal cord. Blood was wiped off with Sugi® absorbent swabs. A pair of magnet-mounted retractors was used to hold back the muscle tissue on the sides to give a clear view of the surgical site. As the vertebral arch was visible, forceps were used to gently lift up the bone from the dura of the spinal cord. In order to not puncture the dura or sever the cord, spring scissors with upwards aiming blades were inserted on the lateral sides of the lamina close to the pedicle. The vertebral arch was incised and the lamina was lifted up to expose the spinal cord underneath. This dorsal laminectomy was repeated one level further cranially at L4.

On both sides the remaining pieces of the pedicles were removed using a Rongeur thereby revealing the DRGs.

The pre-prepared glass pipettes were then placed in a stereotactic frame directly above the surgery site and exactly positioned over the DRG. The DRG was fixated with forceps holding the dura up, as the pipette punctured it and 1µl of the rAAV-*CAG-mOrange* solution was injected.

This procedure was repeated for each of the four exposed DRGs.

At the end of the surgery the spinal cord was examined for any remaining pieces of bone, if necessary removed and the wound was closed (two stitches for the muscle layer and skin sutures). Before and after the surgery the mouse was placed in a padded, pre-heated cage and 500µl-1 ml of NaCl was injected subcutaneously to rehydrate the animal.

The principle of the surgery was the same for control and acute and chronic EAE group, with the exception that chronic EAE animals had already lost some weight at the time of surgery, therefore required less anaesthetic, more careful handling and longer recovery.

All the animals were placed back in their cages.

For pain control during recovery the animals were subcutaneously injected with 100µl of Temgesic (Buprenorphine) immediately after recovery from anesthesia as well as 24h and 48h post-surgery.

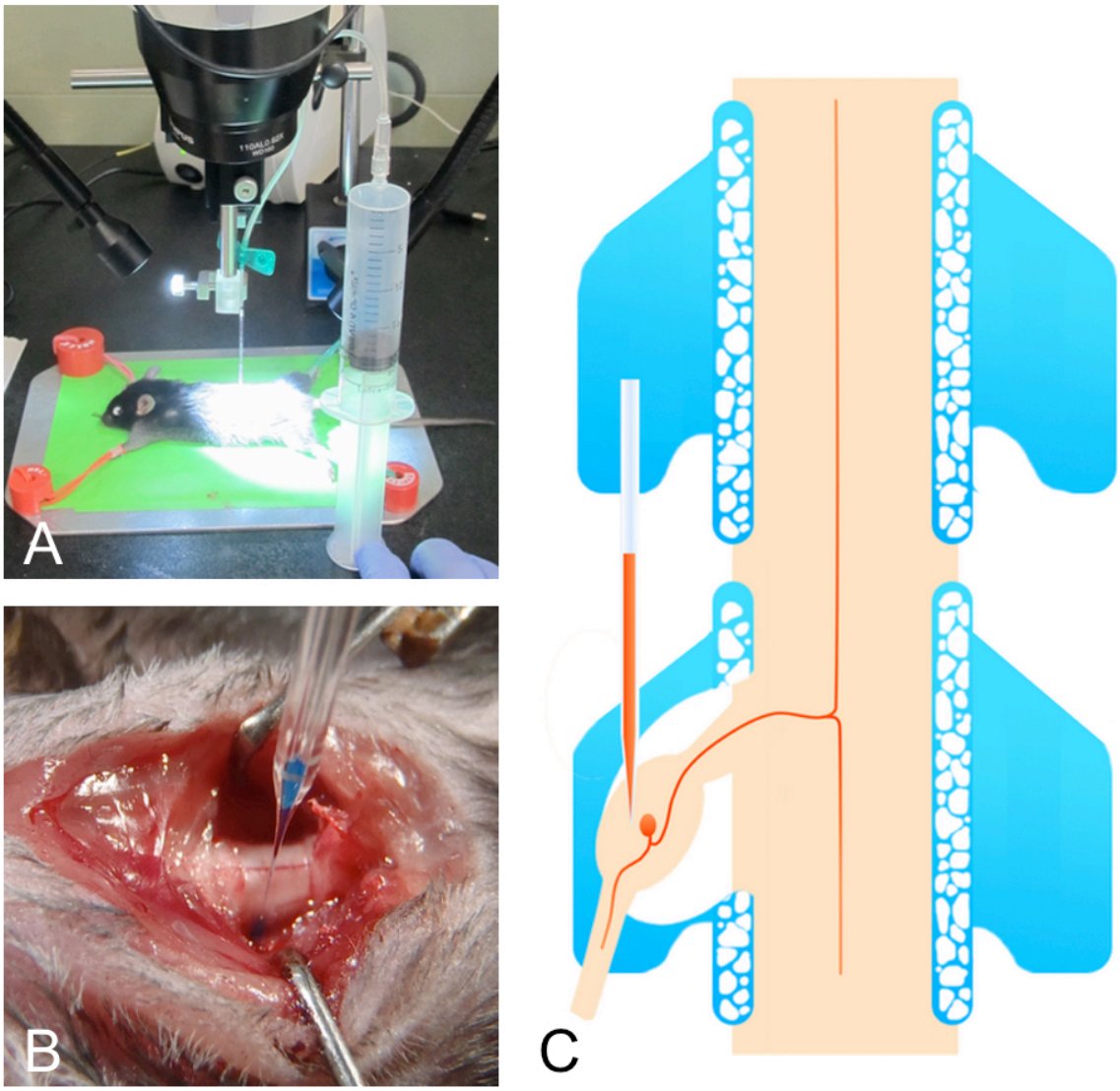


Fig. 3-4 rAAV-CAG—mOrange Injection into the DRG

(A) Setup for DRG injection, *(B)* Exposed spinal cord after laminectomy, the glass pipette punctuates the dura and injection of the virus into the DRG, photograph courteously provided by Fabian Laage-Gaupp *(C)* schematic display of DRG injection and consequent labeling of the sensory axons in the spinal cord with an orange dye

3.2.5 Perfusion and immunohistochemistry

The animals were transcardially perfused on day 21 after surgery with 4% Paraformaldehyde (wt/vol) and post-fixed overnight at 4°C. After fixation the spinal cord was removed and stored in PBS/ 0.1% sodium azide at 4°C. A 3mm piece of the lumbar spinal cord was isolated and incubated in 30% (wt/vol) sucrose in PBS for two days for cryoprotection. The tissue was embedded in TissueTek (Sakura Finetek Europe B.V) solution and frozen at -20°C. Using a cryostat 20µm longitudinal sections were cut.

For immunohistochemistry the sections were inserted into wells (40-50 sections/well) of 6-well plates and washed three times for 10 minutes with 1xPBS on the shaker, then blocked with 5% goat serum (vol/vol) in 0.3% Triton X for 1h. The sections were washed again with 0.3% Triton X (three times 10 minutes on the shaker). The primary antibody against synapsin I (Merck Millipore), a neuronal phosphoprotein which is associated with the cytoplasmic surface of synaptic vesicles (Huttner, Schiebler, Greengard, & De Camilli, 1983) (concentration of 1:500) in a solution of 2.5% goat serum in 0.3% Triton X was added and incubated for 12-24h at 4°C. The tissue then was incubated with the secondary antibody (Alexa-Fluor® 633 goat-anti-rabbit IgG, Alexa-Fluor® 647 donkey anti-rabbit IgG, Invitrogen GmbH) at a concentration of 1:500 and counterstained with a nuclear dye (NeuroTrace ® 500/525 fluorescent Nissl stain, 1:500, Invitrogen GmbH) for 12h at 4°C.

In the end the free-floating slices were washed with 1xPBS and mounted on standard object slide. They were sealed with Vectashield with a cover plate and nail polish to prevent them from drying out. Storage in between scans and evaluation was at -20°C.

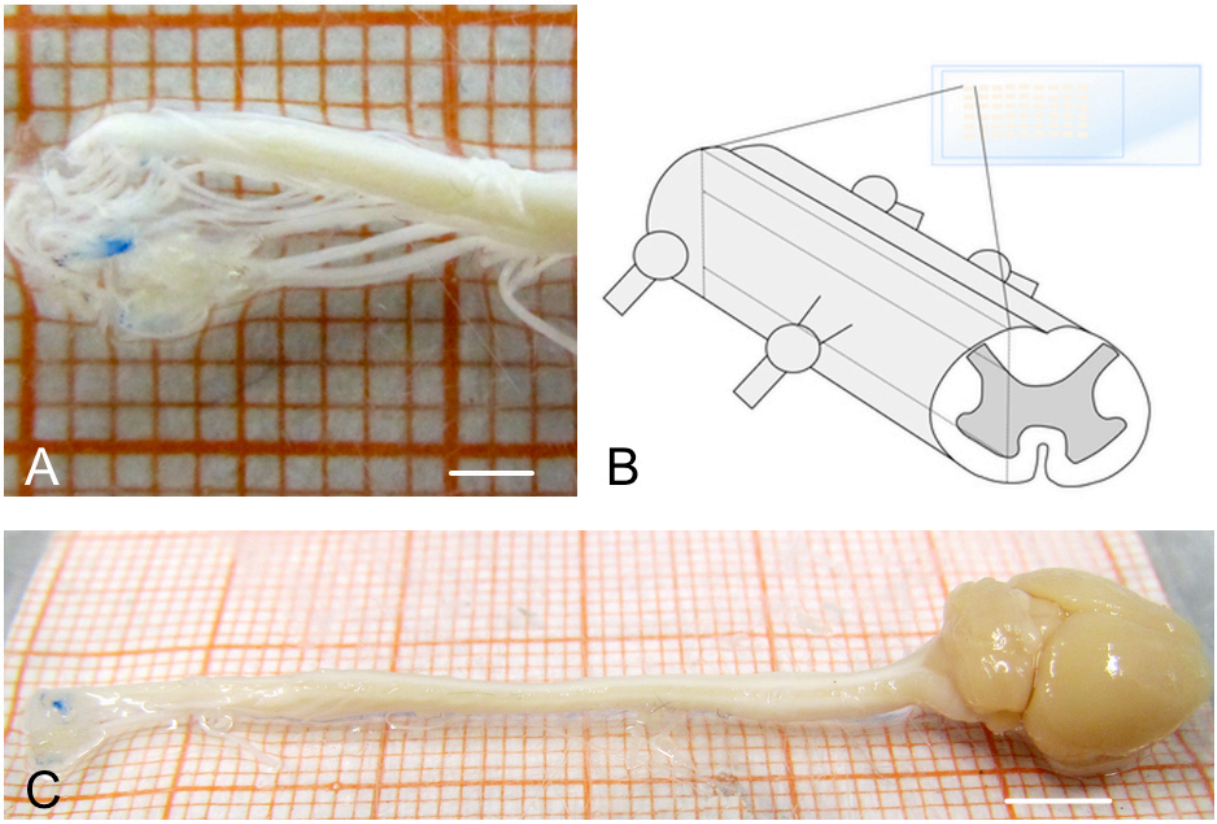


Fig. 3-5 Dissected central nervous system of a mouse
(A) Magnified view of the dorsal root ganglion marked with blue dye in order to verify the success of the injection, *(B)* Overview of the dissected nervous system of a mouse with blue dyed DRG, *(C)* schematic display of the spinal cord part used for analysis (lumbar cord, injection at L5/6), scale bars are 2mm in *A* and 5mm in *B*

3.2.6 Imaging

Sections were chosen for evaluation based on the expression of rAAV-*CAG-mOrange* allowing for the visualization of dorsal column axons with collaterals extending into the gray matter. In EAE tissue, only those sections with densely packed inflammatory lesions in the dorsal column through which rAAV-labeled axons passed were included in the analysis.

The images of the fixed tissue were taken with a confocal microscope with a FV 1000 system mounted on an upright BX61 microscope (Olympus) equipped with x10/ 0.40, ×20/0.85 and ×60/1.35 oil-immersion objectives. Overview images were taken as evidence that the aforementioned criteria were met. To characterize the mitochondria within the distal

collaterals, several high-resolution images (60x, 2.5 zoom) were taken within the gray matter. A z-projection was created at intervals of 0.33 µm.

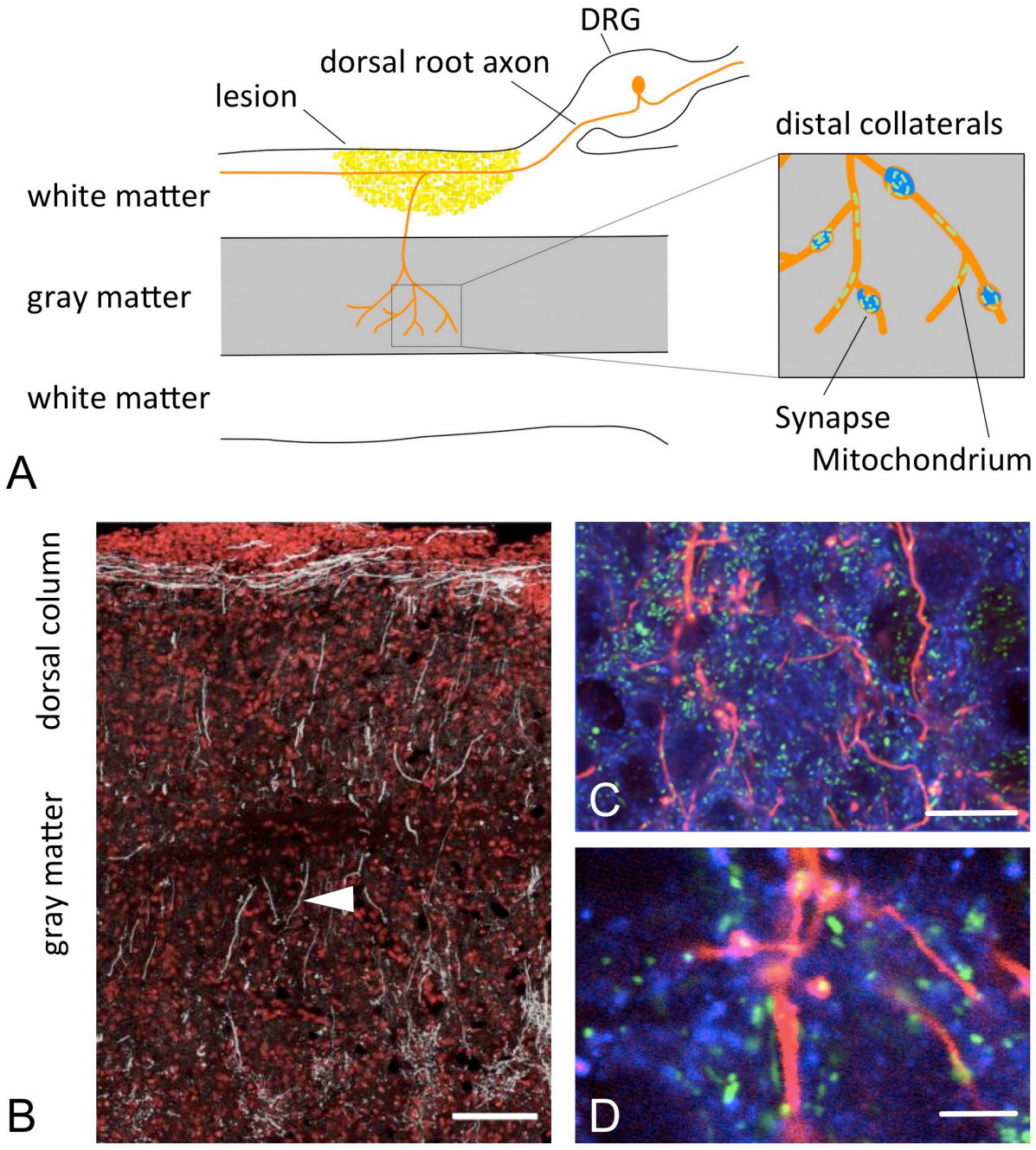


Fig. 3-6 Imaging of the distant collaterals of axons passing through a lesion
(A) Schematic of the scanned section from the spinal cord *(B)* Confocal image of the sagittally cut lumbar

spinal cord of a Biozzi ABH x *Thy1-MitoCFP-S* mouse with chronic EAE (onset+ 21days) showing rAAV-*CAGmOrange*-labeled axons passing through a lesion and branching into the local gray matter (white:rAAV-positive axons; red: nuclei labeled with NeuroTrace 500/525; arrowhead: example of distal axon arbor; mitochondrial channel not shown, from Sorbara et a. 2014). *(C)* High resolution image of collaterals in the gray matter *(D)* Magnified view of the synapsin-positive boutons and mitochondria within the axon (rAAV-positive axons, red; mitochondria transgenic ally labeled with CFP, green; boutons labeled with synapsin antibody, blue) Scale bars are 100µm in *B*, 20µm in *C,* 5µm in *D.*
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3.2.7 Evaluation

The scans were evaluated with the Image J software. Here, images were combined such that rAAV-labeled axons were pseudo-colored in red, mitochondria in green, and synapsin I-labeled structures in blue.

Thirty to fifty collaterals with synapsin I-positive boutons (syn+ boutons) were chosen randomly within a frame and examined for their mitochondrial content excluding synaptic structures. Synapses could be identified by selecting boutons (swellings in the course of the collateral) and comparing with the synapsin I staining, which selectively reveals subunits of synapses.

The collaterals were marked with the segmented line tool to measure the length. The width was measured at three different points with the same axon and averaged in order to calculate the area. Mitochondria in the extra synaptic parts of the axonal arbor were counted so the number of mitochondria per area could be calculated. Additionally data was collected on the content of mitochondria within the synapses.

In a second evaluation collaterals were evaluated that contained both mitochondria and synapsin I-positive structures. Then the mitochondria within the entire segment were counted. Synapsin I-positive bouton structures were counted and their width and length was measured. The approximate volume of a bouton was calculated by assuming an ellipsoid shape.

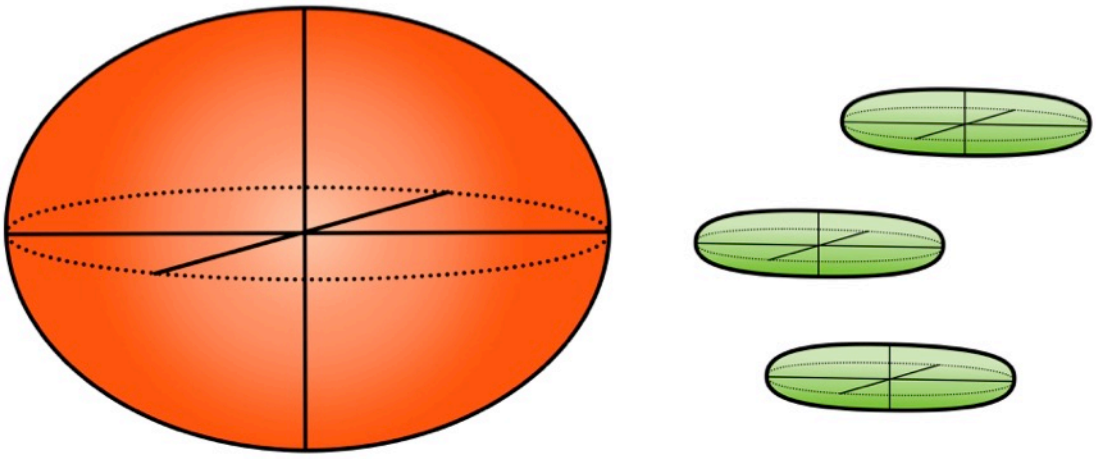


Fig. 3-7 Schematic of Synapse and Mitochondrial Volume calculation

$((x*y*z)/8)*(^4/_3)*\pi$ = volume of an ellipsoid

$(((x*y*z)+(x1*y1*z1)+(xn*yn*zn))/8)*(^4/_3)*\pi$=volume of n>1 ellipsoids

The same equation was used for the volume calculation of the mitochondria (again assuming an ellipsoid shape). Furthermore the number of mitochondria inside the syn+ boutons and between the synaptic structures was counted.

The following values could be deducted from this data: mitochondria (excluding syn+ boutons) per axon area, mitochondria (including syn+ boutons) per area, mitochondria per axon length, mitochondrial volume per syn+ bouton, mitochondrial volume per syn+ bouton volume, number of syn+ boutons per axon length, syn+ bouton volume per axon.

3.2.8 Statistical analysis

All evaluations were calculated with the GraphPad Prism software. The D'Agostino-Pearson normality test was applied to test for normal distribution of all data sets. A nonparametric test was chosen to compare the means between groups, if the data sets were not distributed normally (Mann-Whitney test for two samples, Kruskal-Wallis test with a subsequent Dunn's Multiple Comparison test for two or more samples). Each figure legend indicates the test chosen with its corresponding *P* value if applicable.

All data are represented as the mean ± s.e.m..

3.2.9 Time schedule of experiments

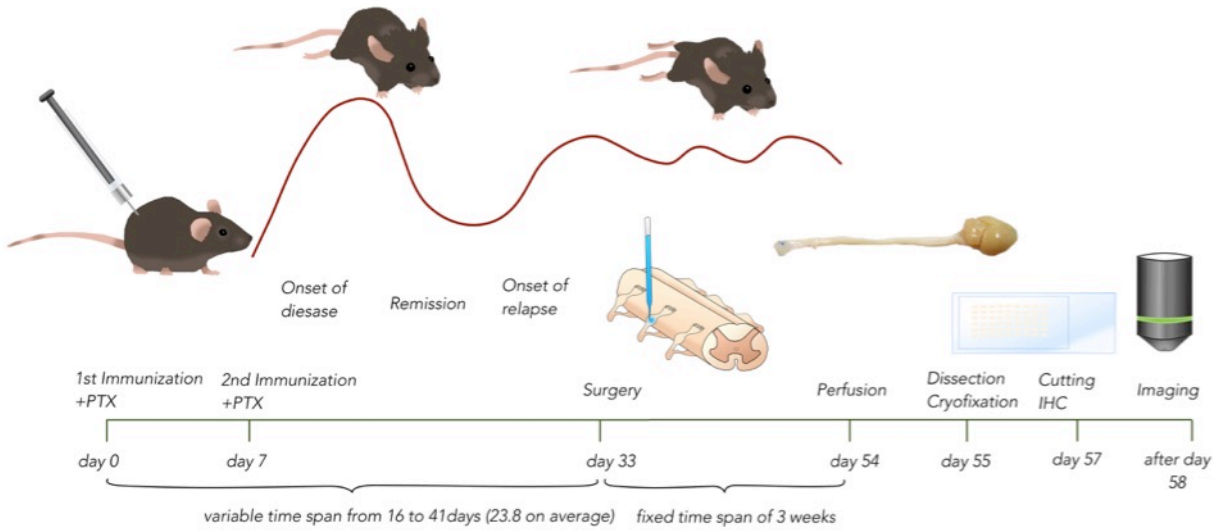


Fig. 3-8 Time schedule for experiments (chronic group)

4 Results

4.1 Establishment of the experimental approach

There were several parameters, which needed to be optimized before I could begin to collect my experimental data and, ultimately, address my scientific questions. The goal was to analyze the mitochondria and synapses in the distal collaterals of neurons in the spinal cord that pass through an inflammatory EAE lesion. Therefore I needed a defined population of axons to analyze fluorescently labeled mitochondria and a way to visualize synapses.

First, a defined population of axons could be labeled by inserting a virus carrying the genetic information for a fluorescent protein that is upon injection incorporated into the neuronal DNA and after transcription and translation in the axon cytoplasm. In order to find the appropriate concentration for the rAAV-*CAG mOrange* virus with a titer of $9x10^{12}$ genome copies/ml, I set up a dilution series whereby I injected into DRGs the following dilutions: 1:2, 1:5, 1:10 and 1:20. The 1:5 dilutions turned out to be best suited and labeled an appropriate amount of axons such that it enabled me to follow a single axon through a lesion and its collaterals into the gray matter.

Dilution	**Concentration**	**Outcome**
1:2	$4.5x10^{12}$	Very dense labeling, distally one could not follow a single collateral due to overlap between labeled collaterals
1:5	$1.8x10^{12}$	Enough axons were labeled to evaluate approximately 30 collaterals per scanned frame, labeling not too dense to identify individual collaterals → chosen dilution
1:10	$9x10^{11}$	Too few axons were stained to evaluate
1:20	$4.5x10^{11}$	Too few axons were stained to evaluate

Tab. 4-1 Dilutions of rAAV-CAG-mOrange injected into the DRG

Second, our laboratory had access to transgenic mouse models with fluorescently labeled mitochondria. To begin, I used *Thy1-MitoCFP-P* mice because this mouse line has previously proven useful in assessing mitochondria in large caliber axons in the dorsal tracts of the spinal

cord (Sorbara et al. 2014, Nikić et al. 2011). To my surprise, this turned out to be unsuitable for evaluation of distal axons. While the large caliber axons in the dorsal column appeared healthy in control animals, the distal branches showed focal axonal dilations and mitochondrial changes. Consequently, I switched to the mouse line, which expresses a lower amount of CFP, and showed no apparent morphological changes of axons in controls, the *Thy1-MitoCFP-S* mice.

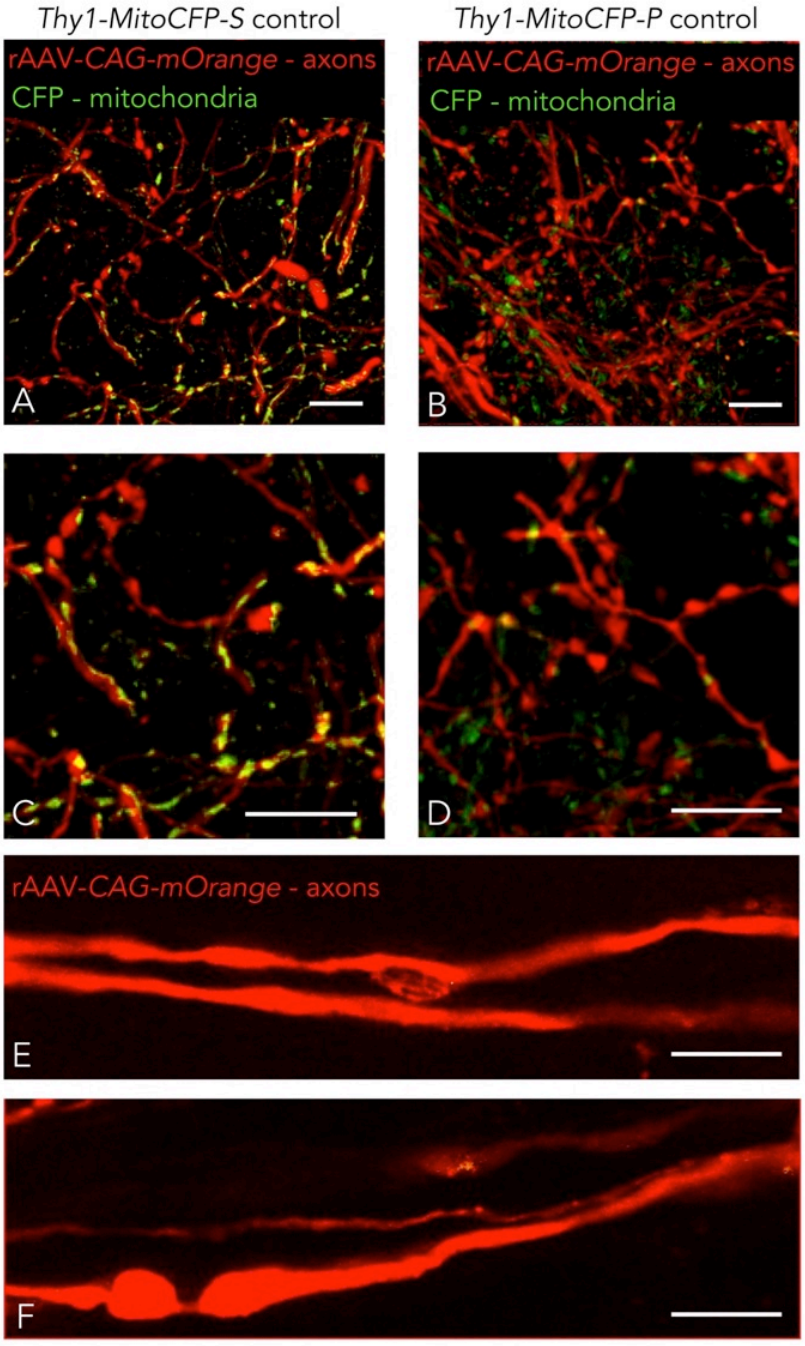


Fig. 4-1 Comparison of axons and mitochondria in *Thy1-MitoCFP-P* and *Thy1-MitoCFP-S* mice
(A)-(F) Confocal 3D-image projection of distal axon collaterals. *(A, C) Thy1-MitoCFP-S* control mouse showing healthy looking axon collaterals with mitochondria *(B, D) Thy1-MitoCFP-P* control mouse showing few mitochondria inside axons, *(E, F)* Confocal images from *Thy1-MitoCFP-P* control mice displaying swelling in large caliber axons stained with rAAV-*CAG-mOrange* in the lumbar spinal cord.
Scale bars are 10µm in *A-H.*

Third, to stain the synapses we tested two possible antibodies directed against synaptic structures: first, an anti-synapsin I antibody (Merck Millipore) directed against a neuronal phosphoprotein that coats synaptic vesicles and secondly an anti-synaptophysin antibody (Cell Signaling Technology) directed against an integral membrane glycoprotein of the presynaptic vescile (Huttner et al., 1983; Wiedenmann, Franke, Kuhn, Moll, & Gould, 1986). For visualization we also had two matching secondary antibodies whose emission colors were far red (633 goat – anti-rabbit, Alexa Fluor) and near infrared (647 goat-anti-rabbit, Alexa Fluor). To test which combination of primary and secondary antibody would be best suited I stained several sections of lumbar cord of two animals with each of the four combinations. The best match proved to be the combination of the anti-synapsin I primary and the Alex633 goat-anti-rabbit secondary antibody, which was subsequently used for all evaluations.

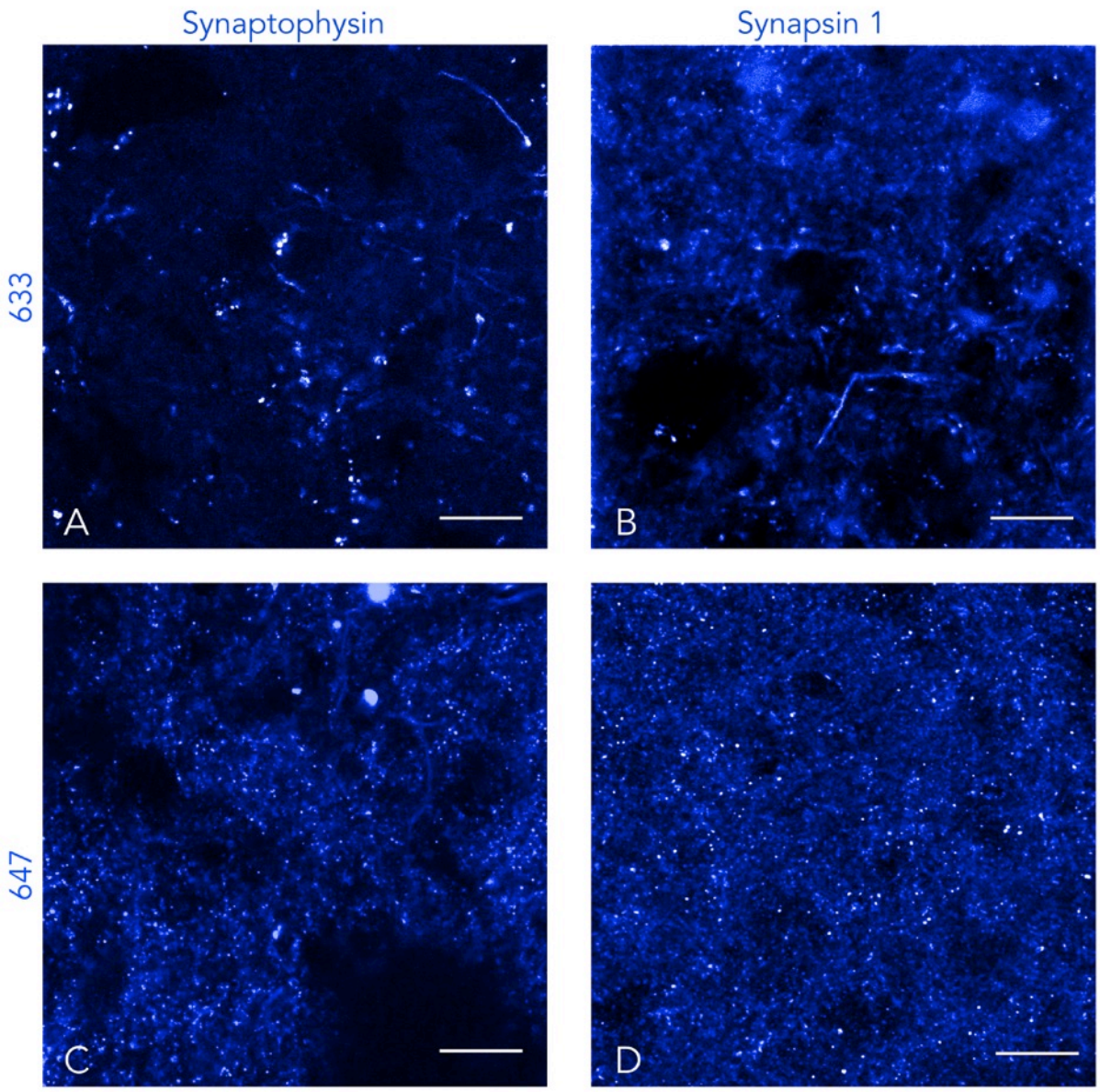
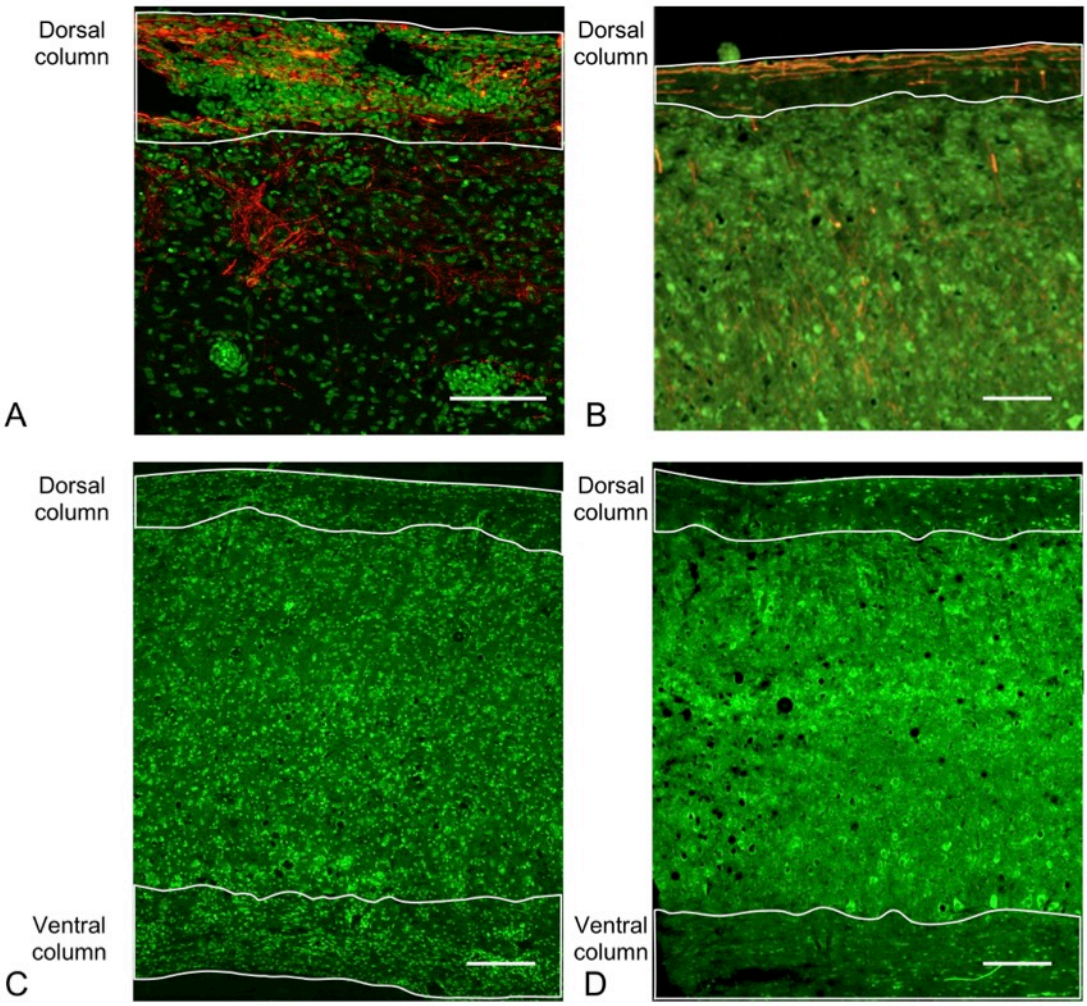


Fig. 4-2 Anti-synaptophysin versus anti-synapsin I antibody comparison
(A-D) Confocal z-projection of gray matter in a lumbar spinal cord section of a healthy *Thy1-MitoCFP-S* mouse stained with four different antibody combinations *(A)* displaying anti-synaptophysin as primary and goat-anti-rabbit 633 as secondary antibody, *(B)* anti-synapsin I and goat-anti-rabbit 633, *(C)* anti-synaptophysin and goat-anti-rabbit 647, *(D)* anti-synapsin I and goat-anti-rabbit 647 gave the optimal staining result to identify synaptic structures.
Scale bars are 20µm in *A-D.*

Finally, since multiple sclerosis is in many cases a multiphasic disease in which an early relapsing remitting phase eventually turns into a chronic progressive phase, it is important to study both

phases when assessing the distal effects of inflammation on the mitochondria in its animal model EAE. Part of my goal was to determine, at what time point during the disease course the transport deficits and mitochondrial changes found in the lesion affect the mitochondrial density in distal collaterals. Therefore I needed to investigate two different groups of animals. One, in which *Thy1-MitoCFP-S* mice were immunized to induce an acute form of EAE. The other a chronic EAE group, which developed an early disease relapse followed by a progressive form of the disease. For this BiozziABH x *Thy1-MitoCFP-S* mice were used. In both cases the EAE groups needed to be compared with respective healthy control animals on the corresponding genetic background.

The part of interest in the spinal cord was primarily the lumbar region corresponding to the level of DRG injection (L4/L5). I intended to analyze the local collaterals that branch off the sensory axons, which pass through the dorsal column. In order to evaluate the effect that inflammation and thus a transport impairment has on distal parts of the neuron, I had to ensure that the axon I was evaluating had passed through an inflammatory lesion. Inflammation was analyzed post-mortem by staining infiltrating and resident cells with a well-established nuclear dye from the NeuroTrace family (Romanelli et al. 2013). Lesions in EAE are characterized by an accumulation of inflammatory cells and can thus be visualized. It became apparent that the acute model (onset of disease +5 days) showed different lesion severity and location than the chronic model. This was especially the case in the lumbar cord. In the acute model microscopically one could identify rather large and pronounced lesions in the dorsal and the ventral column, whereas in the chronic model (onset of relapse +21 days) lesions were smaller and more diffuse in the lumbar part of the spinal cord. In this model I could only identify pronounced lesions sometimes extending throughout half of the diameter of the spinal cord in the cervical spinal cord. In order to proceed, I needed to establish criteria for lesion size that would be included in my evaluation to ensure standardization between samples. Therefore I took overview pictures with tenfold magnification, measured the area of the dorsal column and ventral column and counted the cells within this. Although one could not find as large lesions as in the acute model, nevertheless one could observe a threefold increase in inflammatory cells in the dorsal and ventral column compared to the controls.


Dorsal column
A
Dorsal column
B
Dorsal column
Ventral column
C
Dorsal column
Ventral column
D

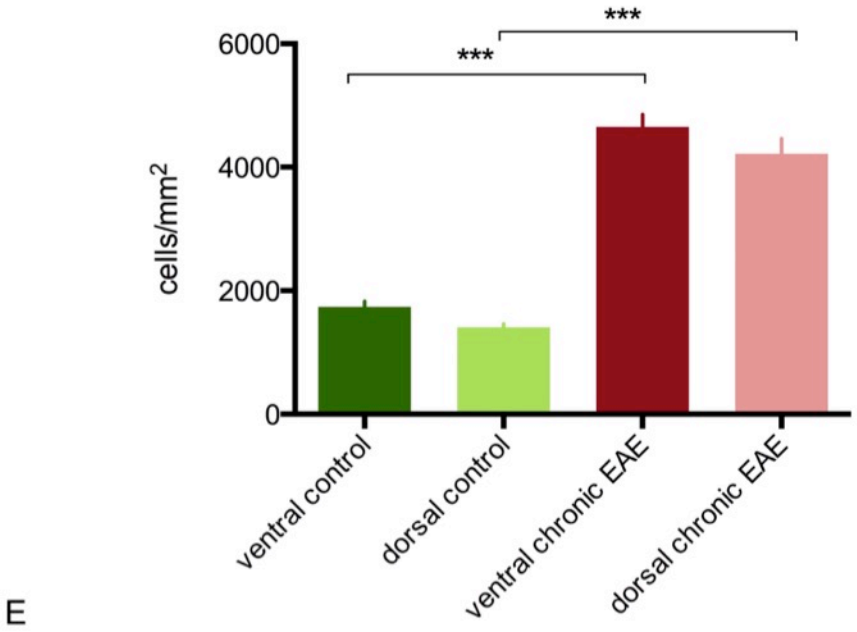

***
***
6000
4000
2000
0
cells/mm2
ventral control
dorsal control
ventral chronic EAE
dorsal chronic EAE
E

Fig. 4-3 Cellularity in dorsal and ventral column in chronic EAE tissue is 3-fold increased
(A)-(D) Confocal z-projection of a longitudinally cut lumbar spinal cord. *(A) Thy1-MitoCFP-S* mouse 5 days after acute EAE onset and its respective healthy control *(B)*, *(C)* BiozziABH x *Thy1-MitoCFP-S* mouse with chronic EAE 21days after onset of relapse and its respective control *(D)*, *(E)* Quantification of the cell density per area in mm^2 within the dorsal and ventral column (n=15-20 frames, 3 mice per group, chronic EAE mice showed a 3 fold increase in cell density in the dorsal and ventral column compared to controls, Kruskal Wallis Test)
Bars represent mean + s.e.m. Scale bar is 100µm in *A*, 200µm in *B-D*, *** $P < 0.001$

As shown in Fig. 4-3 the BiozziABH x *Thy1-MitoCFP-S* mice at 21 days post onset had a significantly higher density of inflammatory cells within the dorsal and ventral columns than those of controls. This ensures for further evaluation that the axons passing through the dorsal matter have passed through sites of inflammation.

4.2 Distal depletion of mitochondria is observed in chronic but not in acute EAE

To examine the effects on acute and chronic neuroinflammation on the organelle content of distal axon segments, I studied mitochondria inside distal collaterals emanating from axons passing through a lumbar, dorsal lesion and branching into the local gray matter at the level of L4/L5. In a first set of evaluations I counted the number of mitochondria per area. For these BiozziABH x*Thy1-MitoCFP-S* mice at 21 days after onset of EAE progression and their respective controls were used. For an acute stage comparison of the disease *Thy1-MitoCFP-S* mice were used at onset of disease + 5 days and compared with their controls. All quantifications of mitochondrial changes in the acute EAE setting have been performed by Catherine Sorbara and Naomi Watson, while I performed the quantitative analysis in chronic EAE.
Together we found a depletion of extra-synaptic mitochondria within the distal arbors of axons in chronic but not acute EAE (mitochondrial density: 0.257 ± 0.017 mitochondria/μm^2 in control axons for acute EAE, n (axon)=219, n(animal)=6; 0.269 ± 0.034 mitochondria/μm^2 in control axons for chronic EAE, n(axon)= 65, n(animal)=6; 0.262 ± 0.021 mitochondria/μm^2 in acute EAE axons, n(axon)= 100, n(animal)=8; 0.142 ± 0.012 mitochondria/μm^2 in chronic EAE axons,

n(axon)=237, n(animal)=8), *** = *P*<0.001). Transport deficits, although striking in the acute phase, do not affect the distal axonal supply significantly (likely because they do not last long enough). In contrast chronic inflammation leads to transport deficits that persist for several weeks and results in a distal depletion of mitochondria.

Moreover, I found that when stratifying the data into a group of small diameter collaterals and another group of large diameter collaterals, by using the mean width from all axons as a cut-off (defined as ≤0.5477µm mean width, large axons as >0.5477µm mean width) the small caliber ones are not significantly more affected than larger caliber axons.

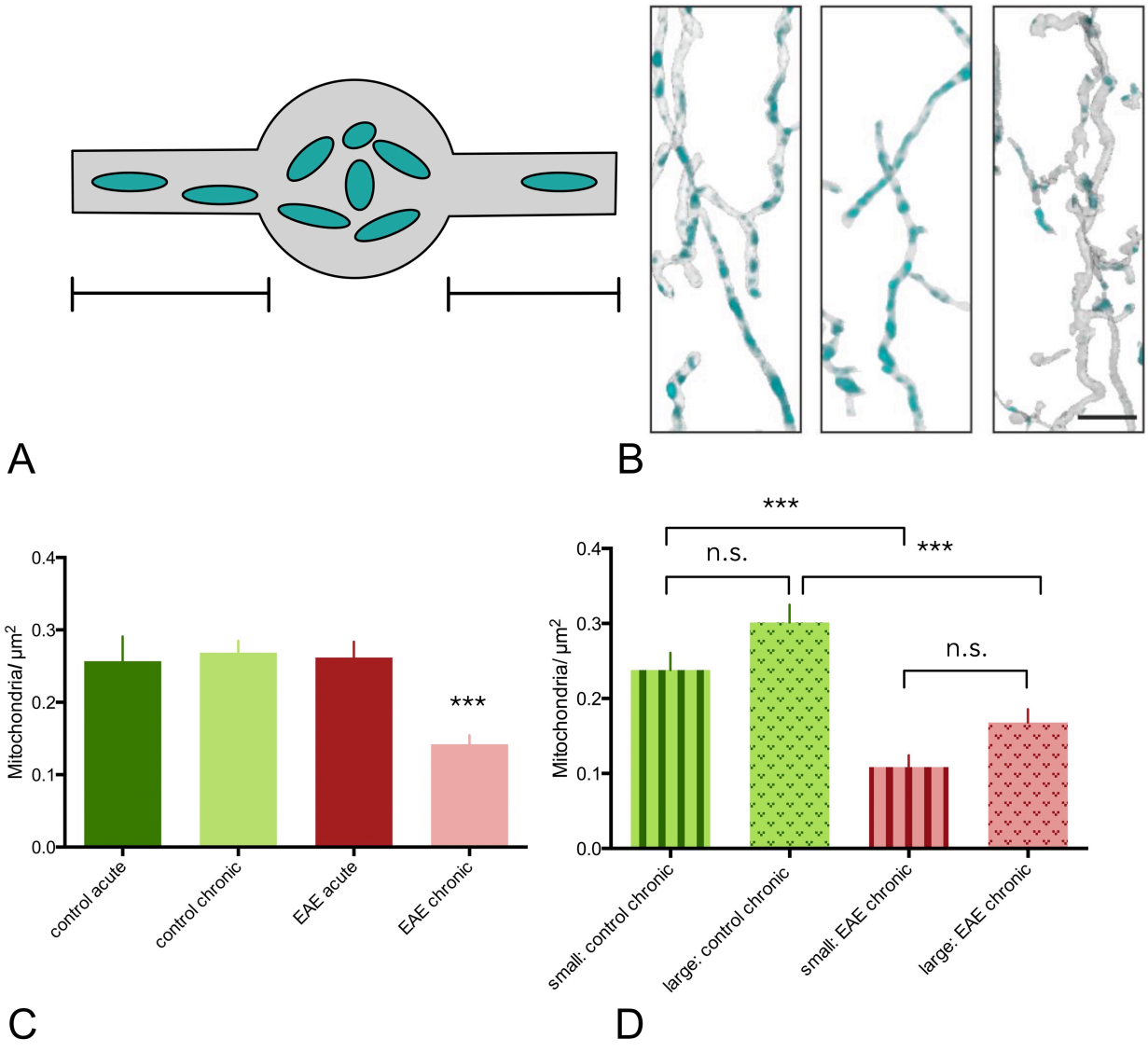


Fig. 4-4 Distal axonal arbors are depleted of mitochondria in a chronic, but not in an acute model of EAE

(A) Schematic of axon fragment (gray) and bouton containing mitochondria (cyan), black bars labeling the area counted for the evaluation. *(B)* Three-dimensional depiction of axon collaterals demonstrating mitochondrial density in control, acute EAE (Onset + 5d), and chronic EAE mice (Onset of progression + 21d), (mitochondria pseudocolored in cyan, axons in gray), image taken from Sorbara et al. 2014, *(C)* Quantification of mitochondrial density in distal axon arbors in acute (Onset+5d) and chronic EAE (Onset of progression + 21d) and their respective control groups *Thy1-MitoCFP-S* (acute model) and BiozziABH x *Thy1-MitoCFP-S* (chronic model) (n = 65-237 axons from 6-8 mice per group; the mitochondrial density is significantly decreased in chronic EAE compared to control group, Kruskal Wallis Test), graph adapted from Sorbara et al. 2014 *(D)* Quantification of mitochondrial density in distal axon collaterals according to axon width in chronic EAE (Onset of progression + 21d) models in BiozziABH x *Thy1-MitoCFP-S* and the respective control group. Small axons were defined as ≤0.5477µm mean width, large axons as >0.5477µm mean width. (n= 103-134 axons from 6-7 mice per group, density is highly significantly decreased in small ($p<0.0001$) and in larger distal axon collaterals ($p<0.05$) compared to their controls, mitochondria in small axon collaterals are not significantly lower than in larger collaterals, Kruskal Wallis Test).

Graph *C* and Illustration *B* are adapted from our publication Sorbara et al. 2014. Rights were obtained from Elsevier. Bar represents mean + s.e.m. Scale bar is 5µm in **(B)** *** $P < 0.001$, * $P<0.05$

4.3 In chronic EAE a larger amount of collaterals is depleted of mitochondria

Having now established that the density of mitochondria is lower in distal axon segments in chronic EAE, I next assessed whether the depletion is widespread among all axons or heterogeneous, with some collaterals containing none at all and others the normal amount of mitochondria.

For this, a random choice of collaterals within a frame was evaluated for their content of mitochondria. The collaterals were qualitatively grouped accordingly, given a score of 0, 0.5 or 1 (more than two CFP-positive structures within the axon fragment and boutons equates to a score of 1, one to two CFP-positive structures within the axon fragment and boutons is a score of 0.5, no CFP-positive structures is given a 0). The percentage of all collaterals not containing any visible mitochondria from the total number of collaterals was calculated. In chronic animals

there was a significantly larger proportion of distal collaterals without visible mitochondria than in controls.

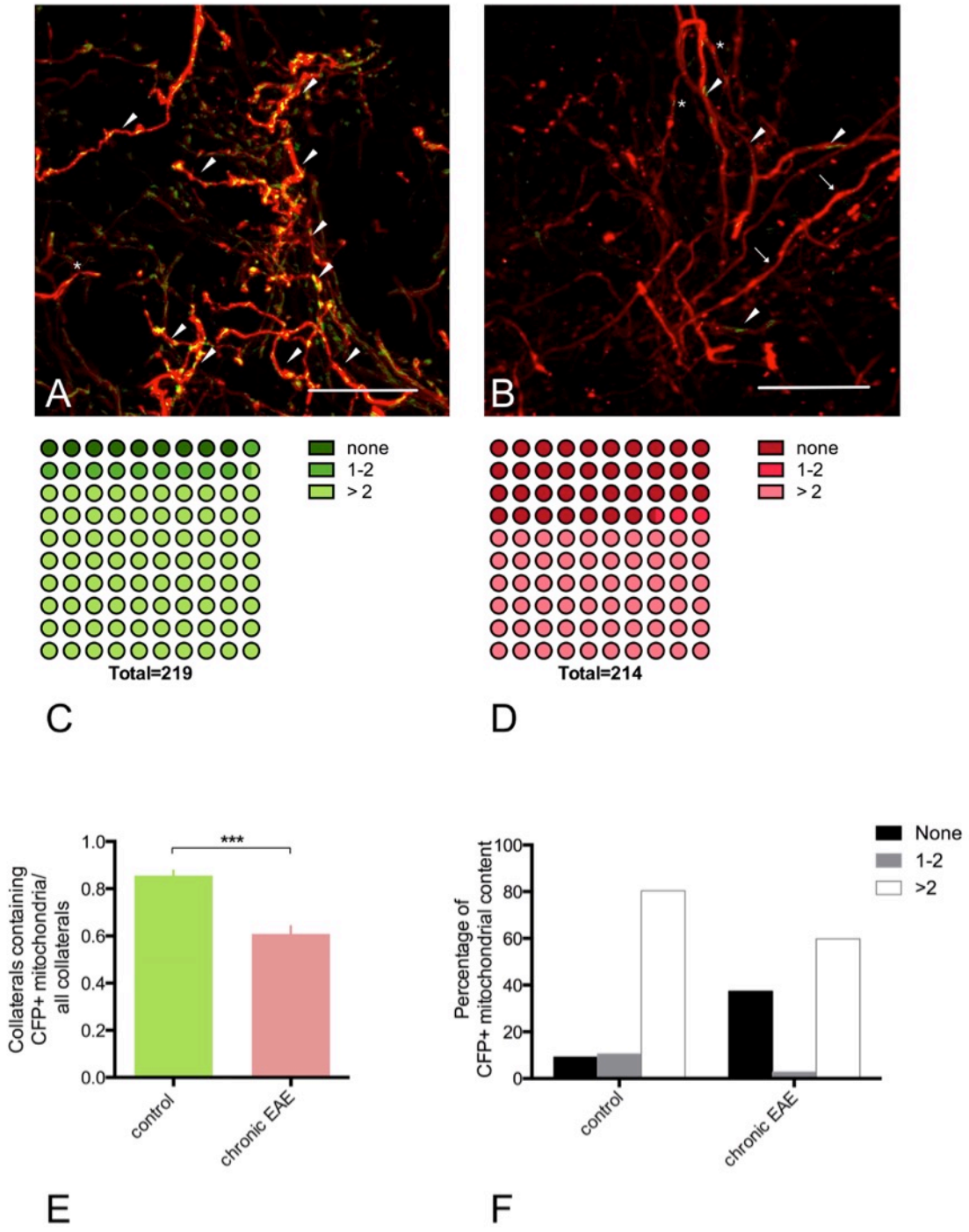


Fig. 4-5 A larger proportion of distal axon collaterals are depleted of CFP+ mitochondria in chronic EAE compared to healthy controls

(A), (B) Three-dimensional rendering of axon collaterals illustrating the proportion of axons containing mitochondria compared to a chronic model of EAE. Arrow heads indicate axons with mitochondria, stars indicate axons with one to two CFP-positive structures, arrows indicate axons without CFP-positive mitochondria *(C)* Pie chart of the percentage of axons containing visible CFP+ mitochondria in healthy BiozziABH x *Thy1-MitoCFP-S* control animals and *(D)* in BiozziABH x *Thy1-MitoCFP-S* chronic EAE (Onset of progression + 21d) models. *(E)* Semi-quantitative data from *(C)* and *(D)* in a column chart (n=211-219 from 6-7 animals, healthy control animals had significantly more axons containing mitochondria than animals with chronic EAE, Mann Whitney test)
Bars represent mean + s.e.m. Scale bar is 20µm in *A* and *B* *** $P < 0.001$.

4.4 Mitochondrial distribution in distal collaterals in chronic EAE is disturbed

I next sought to understand whether the axons that did contain mitochondria in chronic EAE, also exhibited a striking decrease when compared to healthy controls.

Firstly, the total amount of mitochondria throughout the entire visible axon length was counted. This included synapsin I-positive boutons. Interestingly, there was no significant difference in the amount of mitochondria in chronic and control animals.

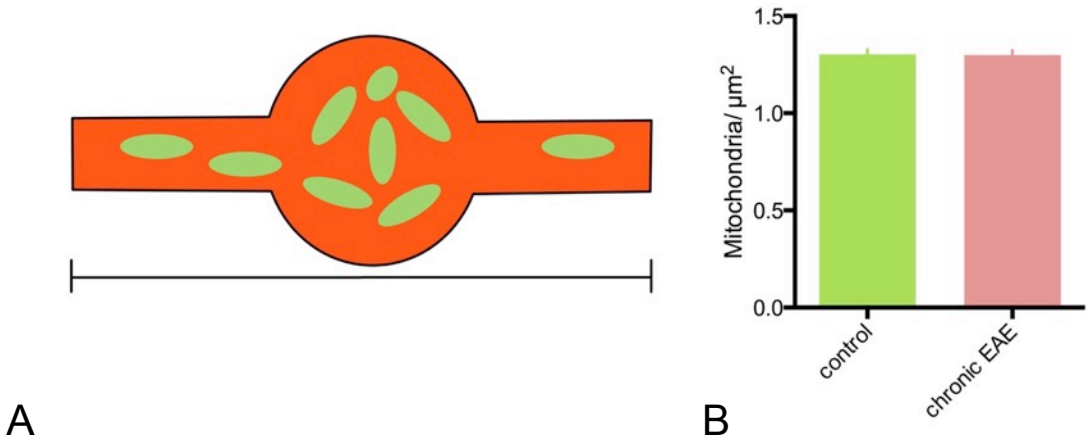


Fig. 4-6 The total number of mitochondria in axon fragments containing mitochondria is similar in chronic EAE and control mice
(A) Schematic of axon fragment and bouton with mitochondria displayed in green, black bar indicating the parts evaluated, *(B)* Quantification of mitochondria per area in µm^2 in chronic BiozziABH x *Thy1-MitoCFP-S* animals and their respective controls in preselected axons containing more than one CFP-positive structure that could be identified as mitochondria, including intra-synaptic mitochondria and

those along its length (n = 518-608 axons, 6 mice per group; no significant difference in density between control and chronic EAE group, Mann Whitney test).
Bars represent mean + s.e.m.

Since no difference in total amount of mitochondria could be identified between control and chronic EAE, I then examined, if there were any changes in the mitochondrial distribution. Prior studies have reported mitochondrial distribution along the axon changes during demyelination and remyelination. However, the distal effects of transport impairment and proximal inflammation on mitochondrial distribution are largely unknown (Zambonin et al. 2011). To answer this, the proportion of the total mitochondria found within the synapsin I-positive boutons was calculated. From this, only a slight difference in distribution could be detected. The trend, however, is consistent. Control animals showed a slightly higher percentage of mitochondria inside the synapsin I-positive bouton than chronic EAE animals, as would be expected given the aforementioned transport deficit

Additionally, the total number of mitochondria inside the synapsin I-positive boutons was counted. This revealed, that controls indeed have more mitochondria per synapsin I-positive bouton than chronic animals. Consequently, in the extra-synaptic area, controls showed a significantly lower mitochondrial density than chronic animals.

In summary, it can be concluded that the distal collaterals of axons passing through a neuroinflammatory lesion, in a chronic stage of EAE, undergo a change in distribution of mitochondria, a potentially early event before the axons are completely depleted. Whereas in the healthy state mitochondria are transported to the place of highest energy demand, namely the synapses. This process appears to be malfunctioning in distal parts of an inflamed axon. The chronic animals showed a higher density of mitochondria in the segments between the synapsin I-positive boutons, but a lower density of mitochondria inside the synapsin I-positive boutons compared to healthy controls.

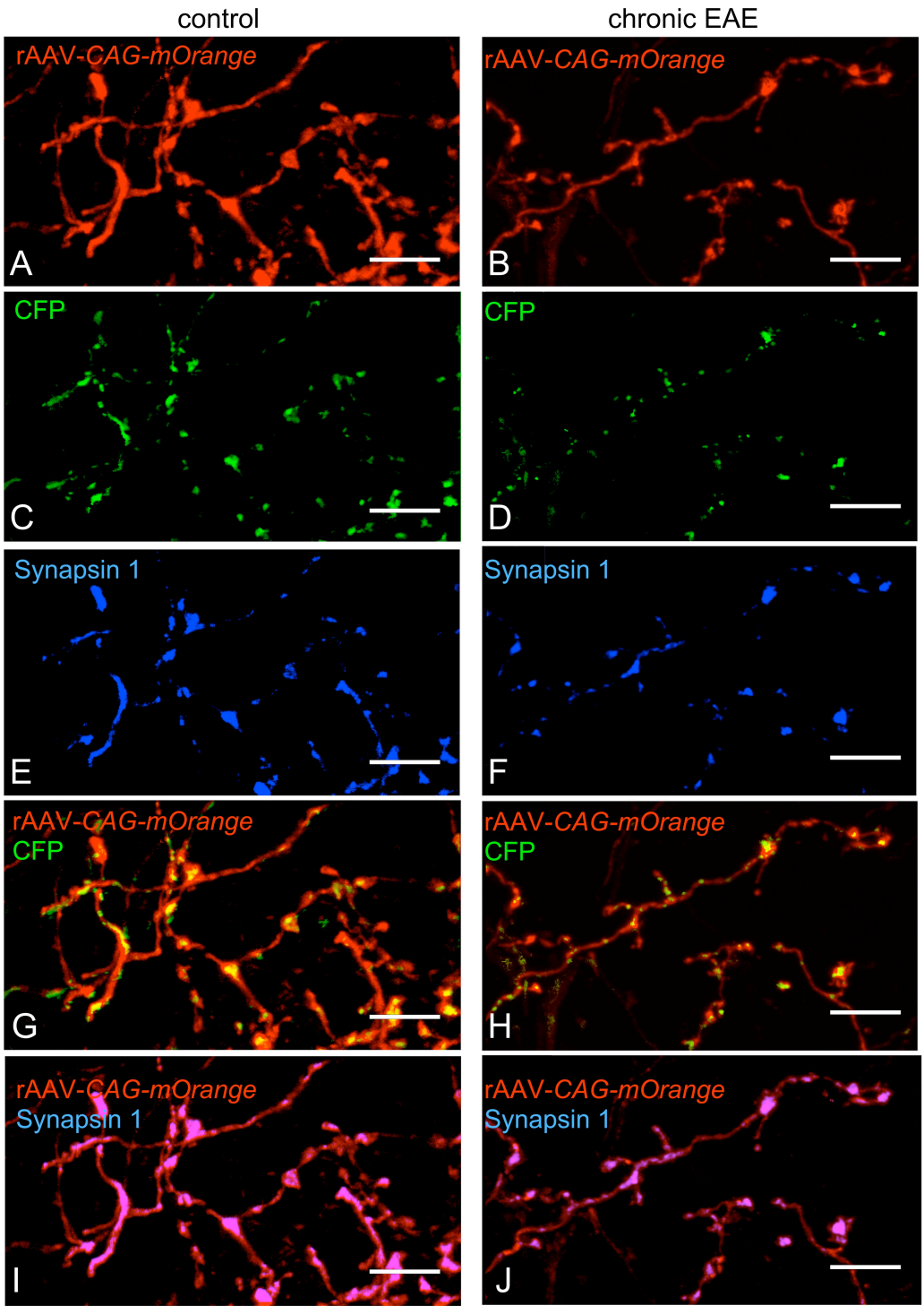
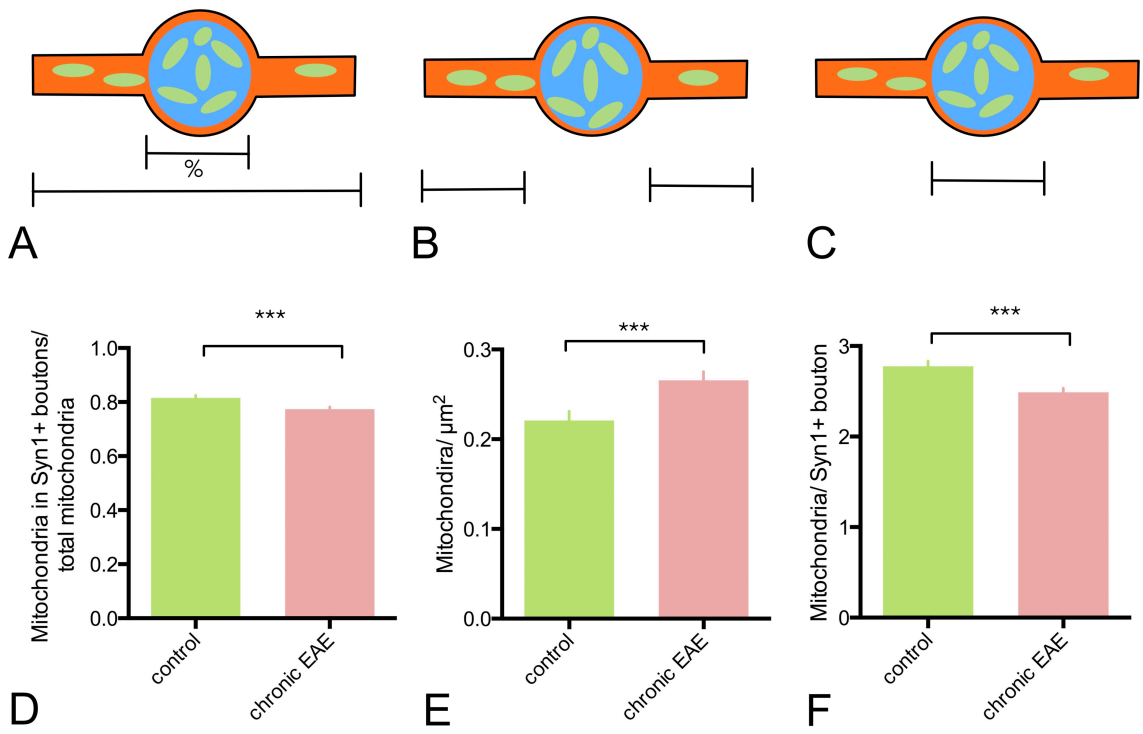


Fig. 4-7 Overview of the different distributions of mitochondria in chronic EAE and controls
(A-J) Three-dimensional rendering of a distal collateral of an axon visualized with rAAV-CAG-mOrange (red) in chronic BiozziABH x *Thy1-MitoCFP-S* mice *(A, C, E, G, I)* and healthy controls, *(B, D, F, H, J)* CFP-positive mitochondria (green) and immunofluorescence staining for the synapsin I antigen (blue) Confocal images at 60x magnification and 2.5 zoom; Scale bars are 8µm in *A-J.*


%
A
B
C
***
Mitochondria in Syn1+ boutons/ total mitochondria
1.0
0.8
0.6
0.4
0.2
0.0
control
chronic EAE
D
***
Mitochondira/ µm2
0.3
0.2
0.1
0.0
control
chronic EAE
E
***
Mitochondria/ Syn1+ bouton
3
2
1
0
control
chronic EAE
F

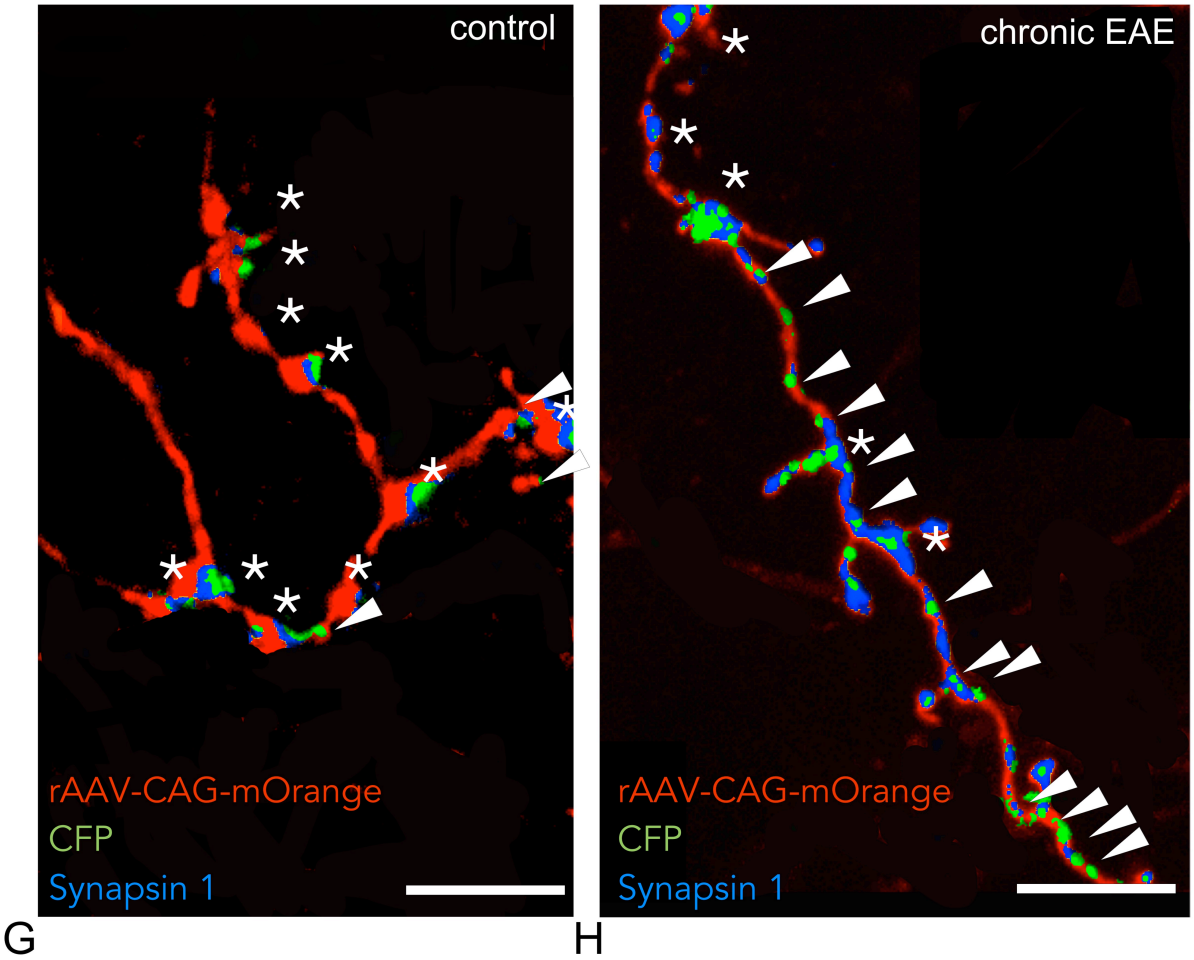

control
rAAV-CAG-mOrange
CFP
Synapsin 1
G
chronic EAE
rAAV-CAG-mOrange
CFP
Synapsin 1
H

Fig. 4-8 Mitochondrial distribution in distal axon fragments is disturbed in chronic EAE
(A-C) Schematics of the distal axon fragment (red) and synapsin I-positive bouton (blue) with mitochondria displayed in green, black bar indicating the parts evaluated; *(B)* is corresponding to *(E), (C)* to *(F), (D)* Proportion of mitochondria inside synapsin I-positive boutons compared to the total amount of mitochondria in distal axons in BiozziABH x *Thy1-MitoCFP-S* animal with chronic EAE compared to healthy controls, evaluated as displayed in *A* (n = 520-609 axons, 6 mice per group; chronic animals have a significantly lower percentage of mitochondria inside the synapses, Mann Whitney test). *(E)* Quantification of mitochondrial density in distal axons excluding synapsin I-positive boutons, evaluated as displayed in *(B)* (n = 547-608 axons, 6 mice per group; chronic animals have a higher mitochondrial density in distal axon fragments, synapsin I-positive boutons excluded, Mann Whitney test); *(F)* Quantification of the number of mitochondria per synapsin I-positive bouton, evaluation as displayed in *(C)* (n=503-607 axons, 6 mice per group, chronic animals have a significantly lower amount of mitochondria inside the synapsin I-positive boutons compared to controls, Mann Whitney test), *(G, H)* three-dimensional rendering of an axon collateral displaying the different distributions of mitochondria in synapsin I-positive boutons and outside in axon fragments. (Immunofluorescently labeled synapses with synapsin I (blue), axons are visualized by rAAV-*CAG mOrange* (red) and CFP+ mitochondria are displayed in green, confocal images taken at 60x magnification and 2.5 zoom)
Bars represent mean + s.e.m. Scale bars are 8µm in *G* and *H* *** $P < 0.001$

4.5 Mitochondrial volume inside the synapse is lower in chronic EAE

Previously, I revealed a decreased number of mitochondria in synapsin I-positive boutons of animals with chronic EAE compared to controls. However the sheer number does not give any information about the actual mitochondrial mass inside the boutons. The volume of the CFP - positive cell organelles within boutons that were labeled with the antibody against the synaptic protein synapsin I was therefore calculated. The mitochondrial mass in chronic animals was significantly decreased compared to healthy controls. To determine if this change in mitochondrial mass has any effect on synapse volume, the volume of the synapsin I-positive boutons was measured. The bouton volume was found to be decreased as well in chronic EAE (Fig.4-10). To detect whether this results into proportionally smaller boutons with less mitochondrial volume or a disproportionately decreased mitochondrial mass, I calculated the ratio of mitochondrial volume and synapse volume, which was found to be decreased in chronic EAE. From this, I hypothesize that in distal axons in chronic EAE, mitochondrial mass decreases

more than the bouton size, which may lead to improper synaptic functioning. Synapses seem to not only decrease in size but also be devoid of mitochondria as illness continues in chronic EAE.

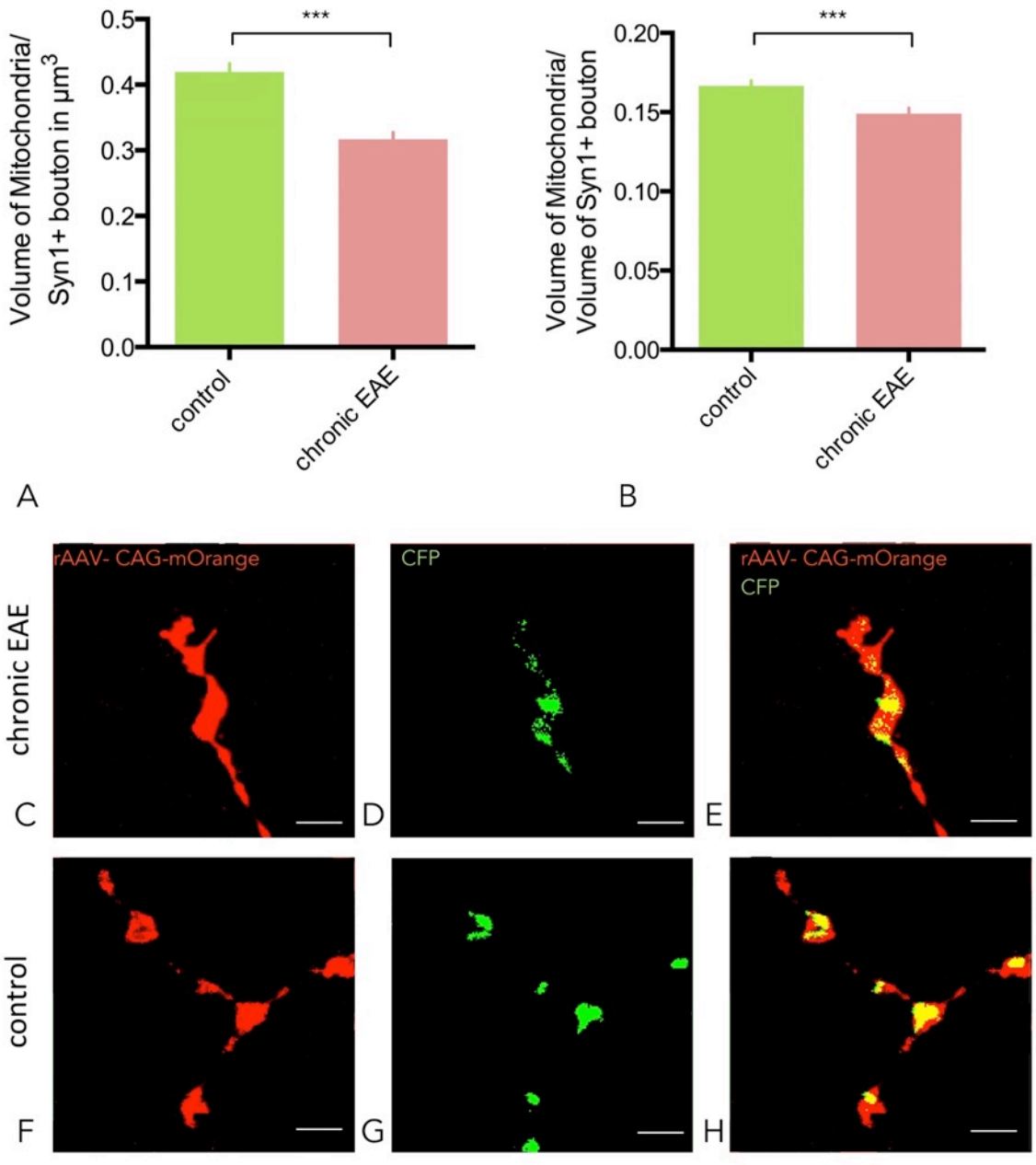


Fig. 4-9 Mitochondrial volume is decreased in chronic EAE boutons

(A) Volume of mitochondria per synapsin I-positive bouton in μm^3 in distal axon collaterals in BiozziABH x *Thy1-MitoCFP-S* animal with chronic EAE compared to healthy controls (n = 502-603 axons, 6 mice per

group; chronic animals have a significantly lower volume of mitochondria inside the synapsin I-positive boutons, Mann Whitney test). *(B)* Ratio of mitochondrial volume to bouton volume in distal axon collaterals (n = 499-608 axons, 6 mice per group; chronic animals have a significantly lower ratio than healthy controls, Mann Whitney test); *(E-J)* three-dimensional rendering of an axon collateral displaying the decreased ratio of mitochondrial volume to bouton volume along the axon fragment in chronic EAE animals *(C-E)* and controls *(F-H)* (axons are visualized by rAAV-CAG mOrange (red) and CFP+ mitochondria are displayed in green, confocal images taken at 60x magnification and 2.5zoom)
Bars represent mean + s.e.m. . Scale bars are 2µm in *C-H* *** $P < 0.001$, n.s. $P > 0.05$

4.6 The number of synapsin I-positive boutons remains unchanged in chronic EAE

Since the mean volume of the bouton is affected by chronic EAE at 21 days after the onset of progression, I examined whether the total number of synapses undergo any changes. For this, the number of synapsin I-positive boutons in the distal axon collaterals was counted per micron collateral length. There was no difference in the number of synapsin I-positive boutons in chronic EAE animals compared to controls. As described above, for this first set of evaluations, a random selection of collaterals within one frame was chosen for evaluation, notwithstanding their content of CFP-positive organelles (mitochondria). Then to closer examine only those axons containing mitochondria, I conducted a second count of all axon collaterals and once again found no significant difference in the synapse number between chronic EAE and control animals.

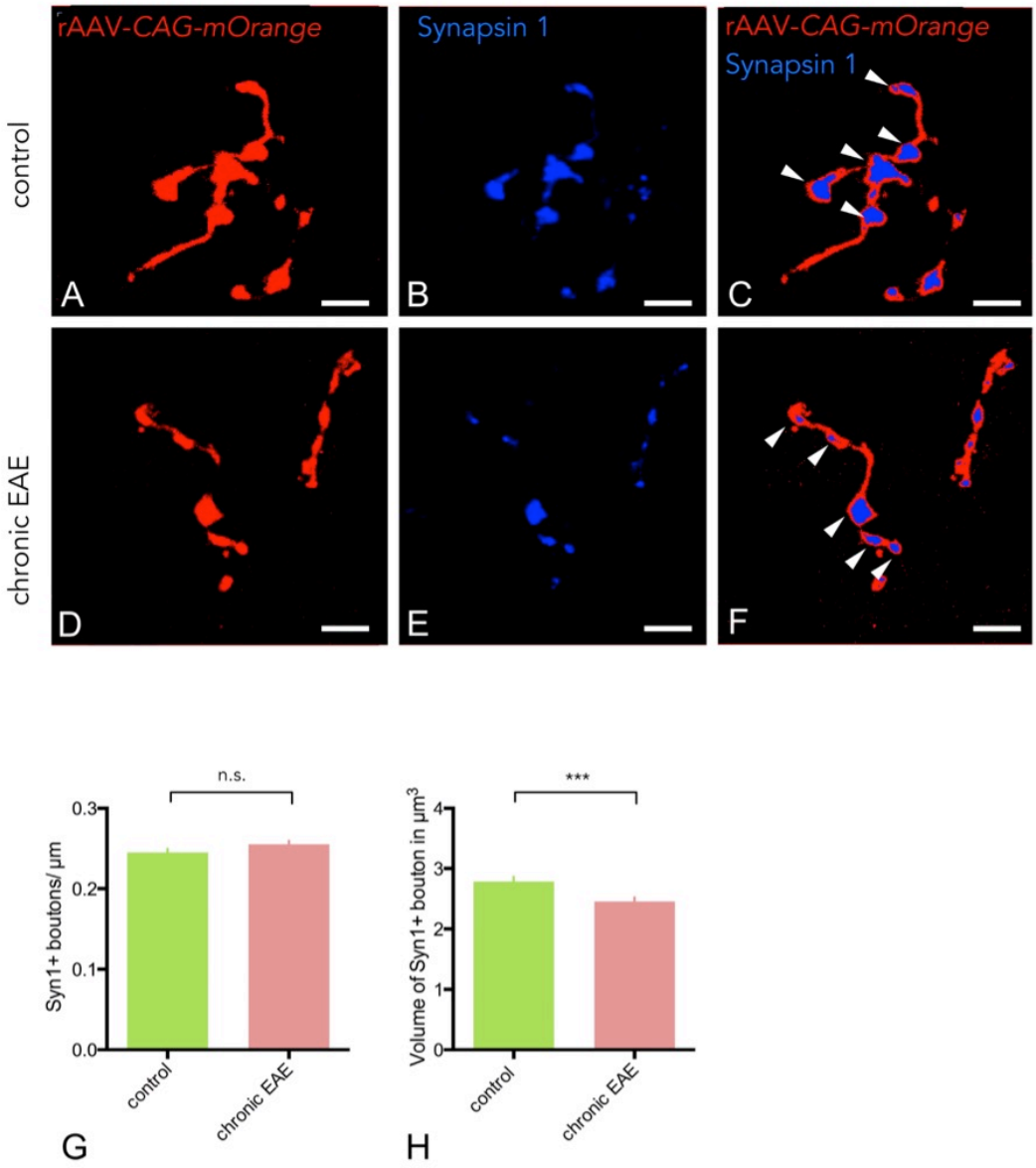


Fig. 4-10 Volume of synapsin I-positive boutons is decreased while density is equal in chronic EAE compared to control animals

(A) - *(F)* three-dimensional reconstruction of confocal images of lumbar spinal cord collaterals (red) *(A, D)* synapsin I staining (blue) *(B, E)* and their overlay *(C, F)* in BiozziABH x *Thy1-MitoCFP-S* animals with chronic EAE *(D-F)* compared to healthy controls *(A-C)* displaying the decrease in volume of the boutons while the number is not affected. *(G)* Quantification of synapsin I-positive boutons per µm in distal axon collaterals containing CFP-positive mitochondria (n=500-606 axons, 6 mice per group, no significant difference in synapsin I-positive bouton frequency could be detected between chronic EAE animals and healthy controls, Mann Whitney test) *(H)* Quantification of mean volume of synapsin I-positive boutons per axon (n=502-603 axons, 6 mice per group, bouton volume in chronic EAE was significantly decreased compared to healthy controls, Mann Whitney test),

Bars represent mean + s.e.m. Scale bars are 4 µm in *A-F* n.s. $P > 0.05$, *** $P < 0.001$

5 Discussion

5.1 The distal effects of the transport defict on mitochondria

This study was aimed at examining the distal effects of the transport block in EAE, an animal model of Multiple Sclerosis. Prior to this study we found an inhibition of anterograde and retrograde transport during acute EAE and a recovery of both during remission.

In the chronic stage of EAE the inflammation is more diffuse than what is observed acutely. Mechanistically, the transport block once prominent within axons of an acute lesion has now lessened as Catherine Sorbara has shown in her set of experiments described earlier. However, full recovery is absent. The anterograde transport was affected more severely than the retrograde. This suggests a net deprivation of several hundreds of mitochondria per day of more distal parts of the axon (C. D. Sorbara et al., 2014). As reported in other neurodegenerative diseases such as Amyotrophic Lateral Sclerosis and Alzheimer's disease, an impairment to axonal transport of mitochondria can lead to a distal reduction of mitochondrial mass (Calkins, Manczak, Mao, Shirendeb, & Reddy, 2011; Marinkovic et al., 2012).

The implications of the pervasive transport deficit for mitochondria in the distal arbor of the neuron, the synapses and the axon terminals in general has been the subject of this work.

5.1.1 Distal mitochondrial depletion in chronic EAE

When examining mitochondria inside distal extra-synaptic parts of collaterals from axons that passed through a lesion, I found a significant reduction in number in chronic EAE compared to healthy controls. However no significant loss of mitochondria could be detected in acute EAE, likely because transport deficits have not lasted long enough to cause significant distal effects. To clarify if the decrease of mitochondria was limited to the smaller axons, I divided the collaterals analyzed into a smaller and a larger caliber group, by using the mean diameter as a cut off value. I could not find any difference in mitochondrial content between the two groups though. Most likely this was because all distal axons I included into my evaluation were already

very small. When analyzing the relationship between the axon caliber and mitochondrial content, I found a directly proportional correlation in control and chronic EAE.

By counting mitochondria in a random selection of distal collaterals regardless whether they contained any CFP-positive mitochondria or not, I included even those axons in my data, which might have contained mitochondria but did not express the fluorescent protein. Consequentially, an axon collateral that did not contain any visible mitochondria could either imply, that the mitochondria were truly depleted by proximal inflammation or they just did not express CFP and could hence not be visualized. To better understand how large the fraction of collaterals was, which did not show any CFP-positive mitochondria I conducted a semi-quantitative evaluation of the mitochondrial count of every collateral in a frame. Those collaterals without any CFP-positive mitochondria classified as 0, those with one to two as 0.5 and those with more than two CFP-positive organelles were given a score of 1. Through this evaluation I found that animals with chronic EAE had a smaller proportion of collaterals containing more than two CFP-positive mitochondria (60%), as opposed to healthy animals (80%). Collaterals entirely without mitochondria represented 9% in healthy animals and 37% in chronic EAE. Furthermore the collaterals with two or less CFP-positive mitochondria made up 10.5% of healthy animals, whereas it represented only a fraction of 2.8% in chronic EAE animals. Consequently the number of collaterals entirely depleted of mitochondria was found increased in chronic EAE while the fractions of axons collaterals with few or more mitochondria are decreased.

Catherine Sorbara examined possible causes of the transport deficit. First, posttranslational modifications of tubulin such as S-acetylation and thyrosinated tubulin have shown to be present in normal-appearing axons, however not to a greater extent than in control axons.

Secondly, as described above Ca^{2+} is an important mediator of mitochondrial transport. Chang and colleagues postulated that not only the cellular but also mitochondrial Ca^{2+} level regulates the speed of the organelle movement (K. T. Chang, Niescier, & Min, 2011). It is likely that the transport deficit is partly due to Ca^{2+} imbalance. Other members of our lab have observed, raised Ca^{2+} levels in 16% of stage 0 axons in lesions in acute EAE, while as was published in our paper 72% of stage 0 axons in acute EAE already display transport deficits (C. Sorbara, 2015; C.

D. Sorbara et al., 2014). Conclusively, Ca^{2+} might not be the dominant factor in controlling transport deficits. However, slight alterations might not have been picked up by the model for Ca^{2+}-sensing, which was used.

Thirdly, subcellular changes induced by NO might be responsible for the transport deficit detected in our project. Sorbara could show that application of an NO-donor could arrest transport, while a NO scavenger resulted in a reinitiation of transport (C. D. Sorbara et al., 2014). This suggests a connection between inflammation and early transport deficits.

There are several possible confounding factors that must be taken into consideration when interpreting these findings: One is that it would be possible that transection of axons in the lesions would result in distal depletion. Here Ivana Nikić described, that of all axons passing through a lesion a proportion of approximately 40% is affected to a degree that FAD (stages 1 and 2) can be observed. Yet she also showed that not only morphological changes to the axon but also on a subcellular level to the mitochondria are occurring very early during the inflammatory process (Nikić et al., 2011). Since those mitochondrial changes can precede axonal changes the axon might appear healthy, while subliminal detrimental changes are leading to axonal degeneration if recovery cannot salvage the axon. However after 30 days only a very small percentage of stage 1 axons remains. Some of them will have returned to stage 0 and recovered and some will have proceeded into stage 2. The vast majority of the distal parts of those axons that have proximally degenerated until day 30 have most likely already undergone Wallerian degeneration. Hence they do not factor into the evaluations of this experimental setting. In fact the confounding effect of transected axons would be more difficult to exclude at the acute time point, where we however did not detect any difference in mitochondrial content. The distal collaterals I was looking at consequently were those axons that survived hence had enough mitochondrial content to withstand the first bout of inflammation.

A second possible confounding factor to be considered is mode of visualization by using transgenic mice. The animal model used in this study was developed by Misgeld and Kerschensteiner (Misgeld, Kerschensteiner, et al., 2007). Since mitochondrial dyes are difficult

to apply in the living animal, this model constitutes a unique opportunity to study *in vivo* mitochondrial dynamics and their pathological alterations. The CFP gene is fused with the mitochondrial targeting sequence of the cytochrome oxidase (COX) subunit 8A gene, regulated by the thy-1 promoter. COX8A is a nuclear encoded gene that transcribes into a subunit of the cytochrome c oxidase complex of the mitochondrial respiratory chain (Chinnery et al., 1999). There are several possible ways for the subunit to be integrated into its destination complex. Classically, the gene is transcribed, translated and subsequently integrated into the respiratory chain complex of the mitochondrion as it is constructed in the soma. The complete functioning organelle is then transported to its destination. With a half-life of approximately 24 days synthesis of mitochondria in the nucleus and transport to the location of insult would be far too slow to react to inflammation. They have the ability to add new parts by fusion or split off parts by fission and depending on energy demand they get redirected. It seems likely that single parts might as well be recruited from the soma as entire organelles to replace broken parts. Recent research on peripheral neurons suggested an alternative way to replace broken parts, in which mRNA is translated distally by a peripheral ribosome machinery (Merianda & Twiss, 2013; Spillane, Ketschek, Merianda, Twiss, & Gallo, 2013). Yoon and colleagues demonstrated that mitochondrial mRNA translation and thereby protein synthesis can also take place outside the soma inside the axon and can thus promote axon survival (B. C. Yoon et al., 2012). Thus broken mitochondria could be repaired in three different ways: by replacing the entire organelle by a newly constructed one from the nucleus, by adding single parts or by distal translation of mRNA, which possibly provides a source for more than one single part depending on how often it is translated and how stable this mRNA is.

Bobylev and colleagues studied mRNA and mitochondrial transport in a model of paclitaxel induced sensory neuropathy and observed a decreased transport for mRNA while the transport of mitochondria was still intact (Bobylev et al., 2015). If this process functioned similarly in central neurons, this would suggest that our observations distally could be partly due to interrupted mRNA transport. Since mRNA does not fluoresce until it is being translated into a protein, some impaired mitochondria could simply not be visualized anymore due to loss of fluorescent protein expression and not due to loss of the mitochondria itself. In this case

however one would primarily expect that the brightness of individual mitochondria might be lower in the affected axons, a change that we did not observe in acute or chronic EAE axons neither at the lesions site nor in more distal parts of the axons.

Lastly, the transgenic animals used do label a large proportion but not all axons. If an axon expresses CFP-positive mitochondria all mitochondria are tagged but in all animals some axons do not express CFP. For the quantitative analysis I have looked at a restricted area of the lumbar spinal cord in six animals per group. Since the branched tree of collaterals in the evaluated area could arise from few proximal axons, a small difference in proximally labeled axons could increase the distal difference. Such difference in the individual labeling patterns should however of course in principle affect all groups analyzed and the low variability of the results observed within groups and also between the two control groups as well as the acute EAE group (see Figure 4-4) make it very unlikely that large differences in the peripheral labeling pattern exist (and act as a confounder of our analysis).

In conclusion, it is thus very likely that the distal mitochondrial depletion I have observed is due to a true lack of mitochondria that results from a transport stop. These results are further confirmed by additional analyses (performed by Catherine Sorbara and Naomi Wagner) that showed a similar decrease in mitochondrial density in distal thoracic parts of the parent axons in the chronic EAE model (Sorbara et al., 2014). In this evaluation only CFP-positive axons were included and a significant difference could be detected.

5.1.2 Distribution and volume control in distal mitochondria

Moreover, my findings suggest that there is a maldistribution of mitochondria distally. When I took a closer look at those collaterals that still contained CFP-positive mitochondria, I found significant differences in distribution, but not in number. In those healthy appearing axons chronic EAE and control animals did not differ in total number of mitochondria per axon segment. In controls however the majority of mitochondria was located inside the boutons, whereas in animals with chronic EAE this ratio was shifted towards extrasynaptic mitochondria. In addition, the mitochondrial volume inside the boutons was found decreased in chronic EAE

compared to healthy controls. To my knowledge there have been no reports on mitochondrial distribution outside of a lesion in EAE yet.

To better understand the implications of these results the following section outlines the crosstalk between mitochondrial volume control and the axonal transport system.

As described above fusion and fission are closely linked with mitochondrial motility. Mutations in fusion proteins can cause an interruption or reduction of transport and a deficient transport can lead to alterations in mitochondrial structure (Baloh, Schmidt, Pestronk, & Milbrandt, 2007; S. Chen, Owens, & Edelman, 2008; Sheng & Cai, 2012). Several examples underline this relationship. Misgeld et al. described stationary mitochondria that were twice the size of motile ones (Misgeld, Kerschensteiner, et al., 2007). Loss of function mutations in the Miro gene cause mitochondrial fragmentation (Fransson, Ruusala, & Aspenström, 2006; X. Liu & Hajnóczky, 2009; Saotome et al., 2008), whereas overexpression leads to decreased fission with elongated mitochondria in Drosophila larval motor neurons and mitochondrial aggregates in dopaminergic neurons (Lee & Lu, 2014; S. Liu et al., 2012; Russo et al., 2009). The dynein-dynactin complex also influences mitochondrial structure. In HeLa cells disruption of the complex leads not only to a redistribution of mitochondria to the nuclear periphery but also to the formation of highly branched elongated mitochondrial structures (Varadi et al., 2004). In a mouse model of Charcot-Marie-Tooth disease with mutated MFN 2, Detmer and colleagues found disturbed mitochondrial distribution; the mitochondria were observed in tight clusters in the axons (Detmer, Vande Velde, Cleveland, & Chan, 2008). Both MFN 2 and Miro have also been shown to be part of the mitochondrial transport unit (Misko, Jiang, Wegorzewska, Milbrandt, & Baloh, 2010). Pink1 and parkin have also been reported to modify fusion and fission. Through PINK/parkin-mediated degradation of MFN and OPA1 proteins, fusion is inhibited and fission promoted through the pathway (N. C. Chan et al., 2011; Kubli & Gustafsson, 2012).

The complex interplay between fusion, fission and transport regulation suggests that deficiency in one area leads to problems in others as well. In this study the distal depletion is likely explainable by the mere decrease in anterograde and increase in retrograde transport rate measured by Sorbara (C. D. Sorbara et al., 2014). The maldistribution however could be

explained in several ways. It might either be an effect of the proximal lesion, which requires more energy to repair the demyelinated or otherwise metabolically disturbed axon and thus redirects mitochondria to retrograde transport to "help out" distally. It could be a result of a distal accumulation of dysfunctional mitochondria due to the proximal inflammation, which are marked for transport back to the soma and are thus swept out of the synapses and into the extrasynaptic parts. It could be the result of a dysfunctional interplay of transport and anchor proteins. The distal transport machinery might be affected similarly as the proximal part of the axon or it could be a compensatory effect to tissue inflammation in normal appearing gray matter.

Metabolic imbalance

Previous studies on mitochondrial distribution showed an increased mitochondrial content during demyelination, remyelination and acute EAE (Kiryu-Seo et al., 2010; C. D. Sorbara et al., 2014; Zambonin et al., 2011). Namely, mitochondria accumulate in lesions where energy demand is high and fail to reach their actual targets - the synapses.

Studies on myelinated axons have shown that mitochondrial transport is arrested by high activity of Na^+/K^+ ATPases and elevated intracellular Ca^{2+} levels (Zhang et al., 2010). Demyelinated axons have to compensate for their dysfunctional conduction by increasing Na^+/K^+ ATPase activity and inserting more Na^+ channels into their membrane. This creates an influx of Na^+, which reverts the Na^+/Ca^{2+} pump to exchange Na^+ for Ca^{2+}. As a result, axonal elevated Ca^{2+} leads to an increase in mitochondrial density in demyelinated parts of the axon (Andrews et al., 2006; Hogan et al., 2009; Bruce D. Trapp & Stys, 2009). Myelination, demyelination and remyelination can individually influence mitochondrial velocity and size, possibly as a rescue mechanism providing continuous energy to the axon (Sheng & Cai, 2012). However, the mitochondria being used to compensate the increased energy demand in the axons might therefore be lacking in the synapses.

Noticeably, Catherine Sorbara showed that transport deficits also occur fairly independently of demyelination, suggesting changes to the transport machinery as one of the earliest events in

EAE and possibly MS pathology (C. D. Sorbara et al., 2014). Demyelination might therefore be merely contributing to an already existing accumulation of mitochondria.

Transport and anchoring machinery

Most likely the transport deficit not only affects the depletion but also the distal distribution. Guo and colleagues demonstrated Miro's role in distal mitochondrial distribution. Fewer synaptic mitochondria and a reduced Ca^{2+} -buffering capacity has been seen in D. melanogaster Miro mutants with impaired axonal transport (Guo et al., 2005). Since Miro is part of the anterograde transport system, which is interrupted in our model, this suggests a causative relationship between the transport deficit and mitochondrial distribution.

As anterograde transport was shown to be permanently reduced, proteins involved in anchoring and allocation to the place of highest energy demand such as syntabulin might also not reach their destination and thus contribute to the difference in distribution. Syntabulin has been identified as an anchoring protein for mitochondria to synapses and as part of the anterograde transport unit (Cai et al., 2005). Studies on syntabulin mutant mice showed reduced synaptic activity, slower synapse recovery after high-frequency firing, and fewer stationary mitochondria within the synapse (Ma, Cai, Lu, Sheng, & Mochida, 2009). A similar result might be reached by inhibition of transport of syntabulin.

Degradation and Evacuation

Not only a functioning supply, but also an intact degradation and evacuation process for impaired mitochondria is necessary for axon homeostasis. The two main mechanisms to achieve that are mitophagy and retrograde transport. Sorbara showed that retrograde transport is elevated in axons proximal to the lesion (C. D. Sorbara et al., 2014). The mitochondrial membrane potential might play a role in transport. Miller and Sheetz reported that depolarized, possibly damaged mitochondria are being transported back to the soma through dynein-dynactin mediated retrograde transport, whereas mitochondria with a high membrane potential are being transported anterogradely (K. E. Miller & Sheetz, 2004). The

mitochondrial depletion of the distal synapses might be due to an increase in dysfunctional mitochondria being transported back to the soma.

For the distal compartment mitophagy is crucial for immediate quality control to limit oxidative damage. As explained in the introduction, Drp1 induces fission in damaged mitochondria, splitting off the healthy part (Losón et al., 2013). Due to reduced membrane potential, PINK1 accumulates on the surface and directs the E3 ubiquitin ligase parkin towards the damaged organelle, which in turn targets the mitochondrion for autophagy by an autophagosome (H. Chen & Chan, 2009). Under mediation by MFN1/2 and OPA1, the healthy salvaged part fuses with other healthy mitochondria parts (Kubli & Gustafsson, 2012). During mitophagy, the outer membrane proteins such as Miro1 and Miro2 are degraded, and PINK can phosphorylate Miro, attract parkin and thus target the damaged part for proteasomal degradation (N. C. Chan et al., 2011). Due to the uncoupling of the mitochondria from its transport system, anterograde transport may be inhibited and wider ranging transport modifications can be suggested (N. C. Chan et al., 2011; Sheng & Cai, 2012; Wang et al., 2011; Yoshii, Kishi, Ishihara, & Mizushima, 2011). Ashrafi and colleagues showed that Parkin and PINK1 were required for the induction of mitophagy in distal axons. PINK1 and Parkin are however also components that have to be transported towards the distal compartments (Ashrafi et al., 2014). Since especially anterograde transport deficits are apparent PINK1, Parkin, Miro and MFN might not be at hand in distal axon in EAE. This possible defect in the degradation system could also account partly for the fact that the number of mitochondria I counted in the axons with CFP-positive mitochondria are not significantly different in chronic and control, the quality might be different though. To examine this further electromicroscopy studies would give better insight into the pathology of individual mitochondria and would not have to rely on CFP tagging.

5.2 The distal effects of the transport deficit on synapses

MS pathology involves inflammation initiated by microglia and macrophages, demyelination stripping the axon of its protective shield and incapacitating it to successfully transmit signals. Mitochondrial changes then deplete the axon of its energy supply and disturbing its Ca^{2+}

homeostasis. Transport deficits amplify this process two-fold: In the lesion they inhibit mitochondrial relocation and their proper distribution to places of energy demand and cause axonal swellings culminating in rupture and eventually axonal transection. Distal from the lesion the local transport block leads to a depletion (and altered distribution) of mitochondria in distal axon segments likely resulting over time in distal axonal and synaptic dystrophy. Indeed such synapse loss appears to be widespread in advanced stages of MS (Jürgens et al., 2016) and disturbances of synaptic plasticity have been linked to clinical progression (Nisticò, Mori, Feligioni, Nicoletti, & Centonze, 2014).

5.2.1 Synaptic pathology in EAE and MS

There are several theories on the causes of synaptic dysfunction and depletion. First, in line with the findings of our work, impaired axonal transport can contribute to synaptic damage and malfunction. Mitochondria are thought to play a key role in synapse integrity and mitochondrial transport to and from synapses has been shown to influence synaptic transmission, density and plasticity (Li et al., 2004). Specifically, mitochondria are able to relocate to dendritic protrusions with increased synaptic activity with the help of Drp1 and OPA1 (Li et al., 2004). Consequently, an alteration of those transport proteins decreased synapse density. In the loss-of-function model of syntabulin, anterograde transport of mitochondria was impaired as well as synaptic function (Ma et al., 2009). This malfunction might be partly due to a specific disability of mobilizing reserve pool vesicles in synapses depleted of mitochondria, which are mainly recruited during repetitive stimulation. This recruitment failure was not due to Ca^{2+} overload but rather to ATP depletion (Verstreken et al., 2005).

In order to secure proper function not only organelles but also synaptic proteins have to be transported to the synapse via axonal transport. In demyelinated areas of hippocampal tissue samples from MS patients, synaptic molecules were decreased, i.e., synaptophysin, synaptotagmin, post-synaptic density protein (PSD95), calmodulin-associated serine/threonine kinase (CASK), glutamate receptors, i.e., AMPA- and NMDA-receptors, glutamate transporters and also KIF1A, another key motor protein in fast axonal transport. This suggests that transport deficits and ensuing synaptic dysfunction in hippocampal neurons can offer a molecular

explanation for deficits in learning and cognition in MS patients (Dutta et al., 2011). Syntabulin mediates presynaptic protein allocation towards the synapse via association with KIF5B (Ma et al., 2009). Ma and colleagues have demonstrated its crucial role in presynaptic function by inhibiting its expression. They found that syntabulin aids in synaptic maturation, sustains basal transmission in mature neurons, prevents synaptic depression during high-frequency firing, and promotes recovery of vesicle and short-term plasticity (Ma et al., 2009). Zhu and colleagues showed a reduction in synapsin I, synaptophysin, and PSD95 immunoreactivity in gray and white matter in acute EAE and during relapse. They also demonstrated during the remission phase of EAE a certain capacity for regeneration of all measured synaptic proteins, albeit not completely (Zhu et al., 2003). This partial remission would also be consistent with the partial recovery of mitochondrial transport that was observed in chronic stages of EAE (Sorbara et al., 2014).

In addition to transport disturbances other inflammatory mechanisms could contribute to synaptic pathology in neuroinflammatory conditions. For example, glutamate excitotoxicity has recently gained attention as a contributing factor in MS pathology. It was shown to be especially harmful in the areas of most synaptic input. Faddis and colleagues showed that glutamate receptor activation could cause dendritic beading and subsequent loss of synaptic connection, whereas upon deactivation synaptic connections would reestablish themselves (Faddis, Hasbani, & Goldberg, 1997; Hasbani, Schlief, Fisher, & Goldberg, 2001). Pitt and colleagues suspected that glutamate excitotoxicity could be an important mediator of axonal damage, oligodendrocyte death and clinical progression in EAE based on the observation that AMPA/kainate antagonists were able to ameliorate clinical symptoms (Pitt et al., 2000). Glutamate has also been shown to correlate with disease activity. In the CSF of MS patients with active lesions on MRI, or during a clinical exacerbation, glutamate levels were significantly elevated compared to those in patients with no signs of active lesions on MRI or during the clinically stable remission phase (Sarchielli, Greco, Floridi, Floridi, & Gallai, 2003).

There are several possible contributors to the glutamate excess in an inflammatory setting. (1) Microglia and macrophages release large quantities upon activation. (2) Demyelinated axons

reverse the Na^+-dependent glutamate transporter, which decreases the uptake capacity for glutamate and possibly even triggers its release into the extracellular space. (3) Astrocytes can not only take up but also release glutamate triggered by Ca^{2+}-dependent or -independent mechanisms (Kostic, Zivkovic, & Stojanovic, 2013). During EAE, glutamate transmission in cerebellar Purkinje cells is increased, owing to prolonged spontaneous excitatory postsynaptic currents (sEPSC). This is caused mainly by reduced re-uptake by receptors such as the glutamate aspartate transporter/ excitatory amino acid transporter 1 (GLAST/EAAT1) on astrocytes, which are downregulated by IL-1β signaling abundant in EAE cerebellum (Georgia Mandolesi et al., 2013) .

Finally, in addition to glutamate release, microglia and macrophages can contribute to synaptic pathology in MS via other inflammatory mechanisms. As described by Stagi and colleagues, the increased production of NO by microglia in a neuroinflammatory lesion can inhibit the anterograde transport of EGFP-tagged synaptophysin and synaptotagmin, as seen in hippocampal neuron cultures. Intact cargo transport of synaptic vesicle proteins is a prerequisite for proper synaptic function. Its inhibition by inflammation might thus lead to synaptic dysfunction (Stagi et al., 2005). A recent study further demonstrated that synaptic density, complement cascade proteins C1q and C3, and increased immunoreactivity for mitochondrial heat shock protein 70 (mtHSP70) are inversely correlated in hippocampal tissue samples from MS patients (Michailidou et al., 2015).

Furthermore, microglial cytokine release of IL1β has been shown to modulate synaptic plasticity. Specifically, it favors long-term potentiation over long-term depression upon repetitive stimulation (Nisticò et al., 2014). It can only be speculated to what extent this is a beneficial or detrimental response. Indeed Chen and colleagues also show evidence for the protective functions of microglia. They propose that due to stripping of inhibitory synapses, neuronal synchronicity is increased, and neuronal pro-survival molecules (Bcl-2, Mcl-1, pBAD) and neurotropic factors (BDNF, FGF-2) are released (Z. Chen et al., 2014). Zhu and colleagues were able to demonstrate extensive dendritic beading in the white matter of rat spinal cords during acute EAE, and a recovery during remission, suggesting that dendritic damage is less

dependent on demyelination but rather driven by inflammation and glutamate accumulation (Zhu et al., 2003). However the same changes could not be found in gray matter, although similar levels of inflammation were observed (Zhu et al., 2003). While there was less beading the gray matter showed similar depletion of presynaptic proteins such as synapsin I and synaptophysin, which were pervasive and longer lasting than the beading (Zhu et al., 2003). Likely the depletion of synaptic proteins was due to transport deficits. Accordingly, synapses are not only deprived of mitochondria, but also of essential synaptic proteins.

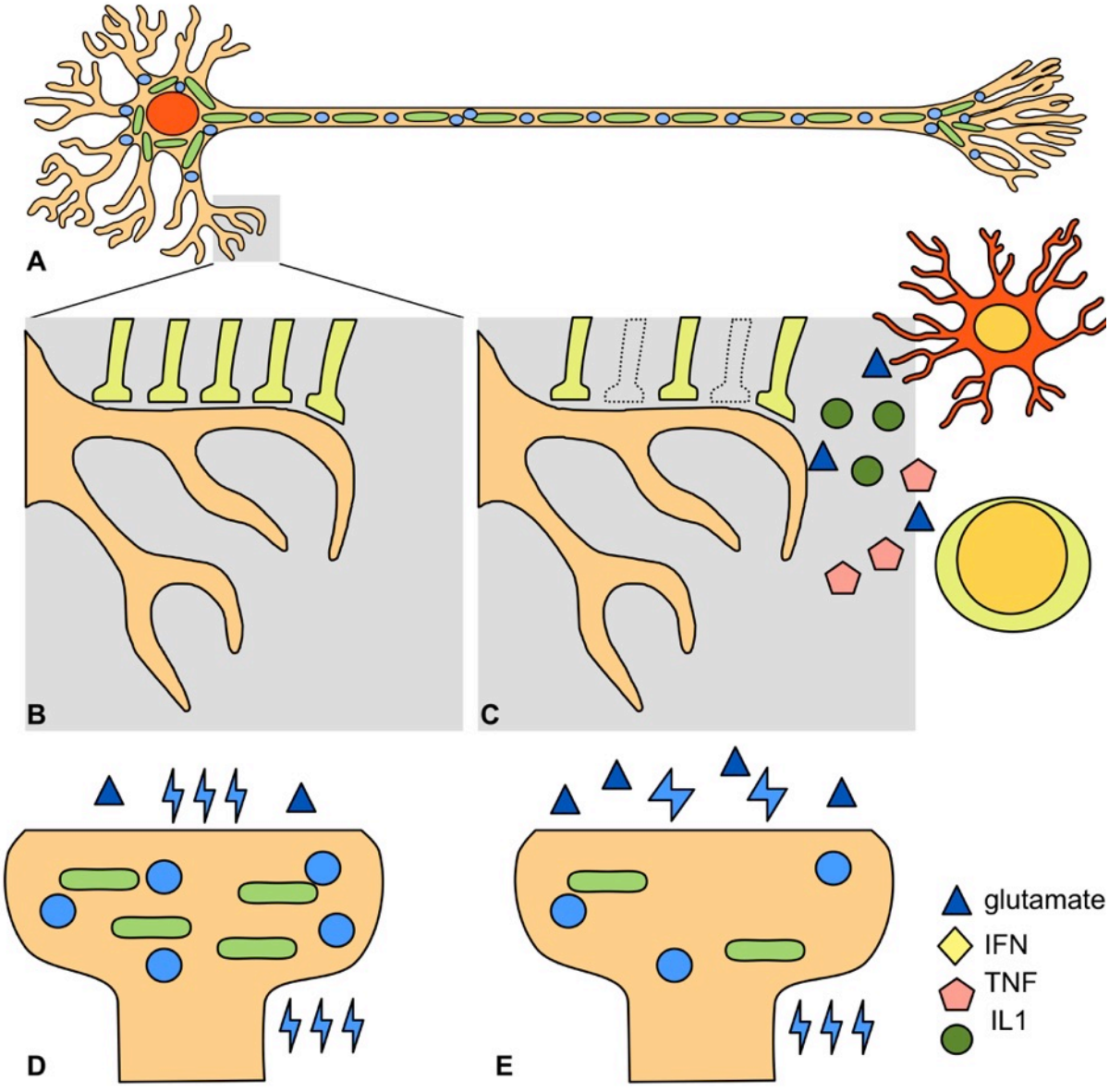


Fig. 5-1 Synapse pathology in MS

(A) Mitochondria (green ovals), synaptic proteins and neurotransmitters (blue circles) are being transported towards the synapse along the axon, during inflammation transport might fail and the synapse is undersupplied, healthy state displayed in *B* and *D*, inflammatory state displayed in *C* and *E*. Microglia, macrophages and T-cells secrete inflammatory mediators such as glutamate, IFNγ, TNFα and IL1, that induce a thinning of synaptic contacts *(C)*, glutamate excitotoxicity leads to prolonged EPSCs, due to transport deficits the synapse is deprived of mitochondria *(E)*, which leads to failure of reserve vesicle mobilization. Synaptic currents are depicted as blue arrows. Adapted partly from (G. Mandolesi, Grasselli, Musumeci, & Centonze, 2010; Sheng & Cai, 2012).

5.2.2 Synapses last longer than their content

In the second part of this study I focused on the implications of the transport deficit for the synapses in the gray matter. The structures analyzed were protrusions along the axon collaterals staining positive for synapsin I. I found the bouton volume along with the mitochondrial volume inside these boutons to be decreased in chronic EAE compared to controls, albeit not quite proportionally since the ratio of mitochondrial volume to bouton volume was also lower in chronic EAE. This supports my results on decreased number of mitochondria inside the boutons in chronic EAE. Furthermore I compared the number of synapses in chronic EAE and control animals and found no significant difference between the two groups.

Synaptic pathology is a difficult subject to address by means of immunohistochemistry, not only because staining might be inconsistent, but also because function is not directly deducible from appearance. I have used an antibody against a presynaptic protein, synapsin I, as explained in detail above. Immunohistochemical staining results are largely dependent on timing, temperature, exposure to light and also tissue treatment beforehand. Additionally once the staining procedure is finished, over time and after each freeze-thaw cycle the antibody fluorescence fades. Finally during scanning exposure time and laser settings should remain the same throughout the entire experiment. Since I could not guarantee those prerequisites, I did not include any data on staining intensity. Better methods for quantification of synaptic function would be the patch clamp method that measures ion currents, by detecting the postsynaptic current (PSC), or by the synaptic release probability- the likelihood of vesicle fusion after the occurrence of an action potential at the presynaptic bouton (Branco, Marra, &

Staras, 2010). With immunohistochemistry one can only infer information about the performance of the synapse.

What can be assessed fairly well by means of immunohistochemistry is bouton size. There are only few and inconsistent publications on regulation of bouton size in the CNS. One stated that inactivity leads to increased bouton size in hippocampal neurons due to accumulation of vesicles (Murthy, Schikorski, Stevens, & Zhu, 2001). Vice versa a lack of vesicles or organelles could decrease synaptic size, as our study is suggesting. Grillo and colleagues stated that rather size fluctuations than the size itself was altered in aging mouse cortex (Grillo et al., 2013). Examining my own results for fluctuations reveals that the variance of synapsin I-positive bouton volume in chronic EAE mice is not different to controls (F-Test of synapsin I-positive bouton volume: control SD = 1.744, chronic SD = 1.66, coefficient of variation control: 62.63%, chronic: 67.67%, the variances are not significantly different $P>0.05$).

Mhatre and colleagues reported altered synaptic morphology, a reduction in total number of synaptic connections (boutons) and decreased mitochondrial intensity in a *Drosophila melanogaster* AD model (Mhatre et al., 2014). Contrary to the results in this model of AD, in this study I have not found a significant difference between the number of boutons per collateral between the chronic and control group. This does not exclude ongoing synaptic pathology though. Ziehn and colleagues quantified synapse density by determining the amount of synapsin I-positive punctae within an image (Ziehn, Avedisian, Tiwari-Woodruff, & Voskuhl, 2010). They found a reduction of synapsin I-positive punctae in hippocampal segment CA1 in EAE. When examining my synapsin I images, I found a significant decrease of mean gray value, when comparing chronic EAE scans with controls. This result could not be utilized and is not shown here due to too many confounding factors such as different time points of staining and parameters of scanning. Nevertheless one can speculate that due to transport deficits not only mitochondria are lacking in the distal arbors of axons passing through lesions, but also pre-synaptic proteins such as synapsin I. As Stagi et al. showed, inflammation can inhibit the anterograde transport of synaptophysin and synaptotagmin and Zhu and colleagues demonstrated the reduction of several synaptic markers with the most pronounced effect in

the white matter and ventral gray matter of the lumbar spinal cord in acute EAE and upon recovery (Stagi et al., 2005; Zhu et al., 2003).

My results suggest that there is a distal mitochondrial deprivation of the synapses possibly due to the proximal transport dysregulation. One can speculate that synaptic proteins such as synapsin I might be decreased as well. Since the content of the bouton is vital for synaptic function, one can deduct that the lack of mitochondria might be an indirect indicator for synaptic dysfunction. Since I could not find a difference in number of boutons, one can postulate that this synaptic pathology begins with a deprivation of their content and a decrease in volume. It is likely that as pathology continues number of synapses would diminish as well. Figure 5-2 displays a synopsis of the results of this study.

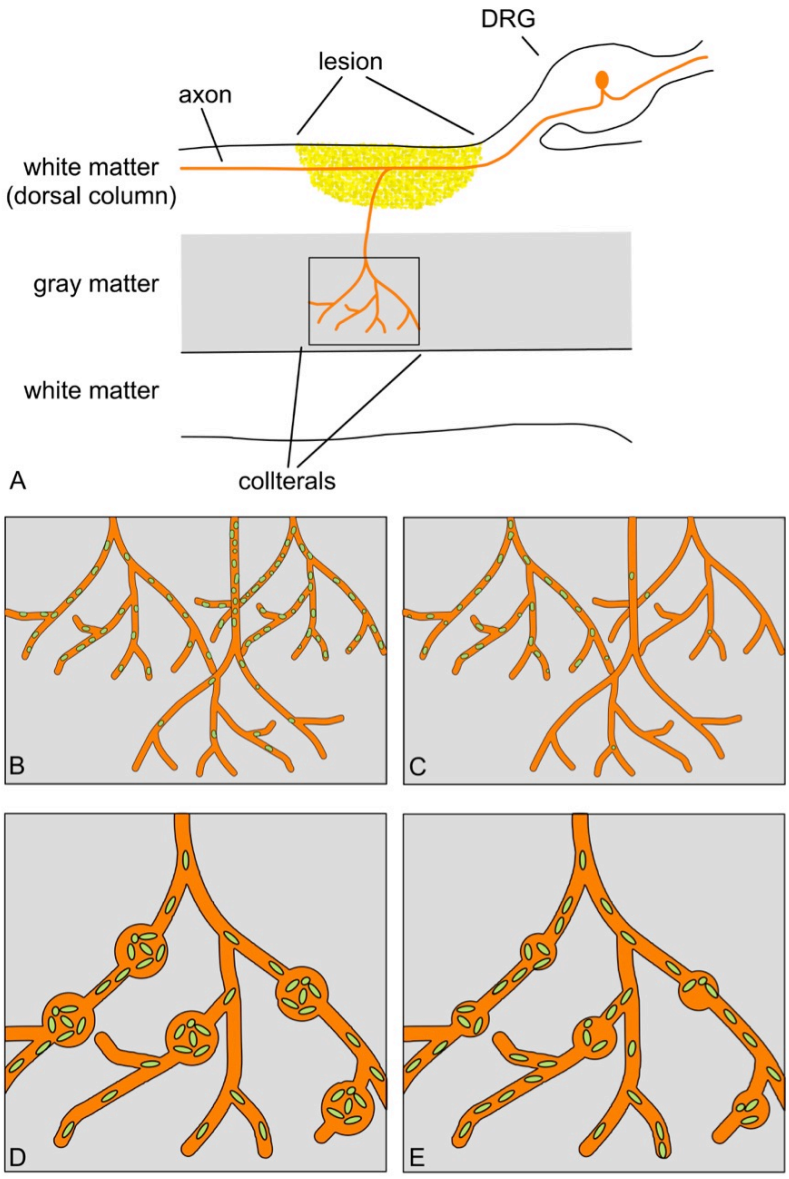


Fig. 5-2 Summary of the results

(A) Schematic displaying the experimental setting *(B, C)* there is a distal deprivation of mitochondria in the distal arbors of axons passing through a lesion in chronic EAE *(C)* compared to healthy controls *(B),* more collaterals show no CFP-positive mitochondria at all *(D, E)* distal collaterals in chronic EAE *(E)* that contain CFP-positive mitochondria display less mitochondria in the synapsin I-positive boutons and more in the extra-synaptic parts compared to control *(D),* bouton volume is also decreased, while the number is not significantly different.

5.3 Gray matter pathology – the uncommon suspect

The distal axonal parts that were examined in this experiment were part of the normal appearing gray matter (NAGM). NAGM also exhibits neuronal injury independent of demyelination, similar to findings in NAWM (Magliozzi et al., 2010; Witte et al., 2014).

Bo, Van Horssen, and their colleagues have shown that subpial gray matter demyelination can be extensive in progressive patients, despite lacking lymphocyte infiltration and BBB damage (Bø, Geurts, Mörk, & van der Valk, 2006; Bø, Vedeler, Nyland, Trapp, & Mørk, 2003; van Horssen, Brink, de Vries, van der Valk, & Bø, 2007). The lesions were associated with axonal and dendritic transections, demyelination, neuronal apoptosis, decreased synaptic density and glial cell loss (Bø et al., 2003; van Horssen et al., 2007; Wegner et al., 2006). On MRI scans, cortical thinning could be detected (Calabrese et al., 2007). Moreover, gray matter atrophy is probably associated with long-term deficiency and occurs early in the disease (Calabrese et al., 2007; Fisniku et al., 2008; Rudick, Lee, Nakamura, & Fisher, 2009).

Looking at the clinical picture the chronic EAE mice exhibited, I found possible coherencies between my chosen parameters of gray matter pathology and disease score. Comparing the mean values of mitochondrial and bouton volume of each animal with its disease score, the data suggests that both are correlated with disease severity. Animals with EAE scores of 3.5 and 3 had smaller mean values for mitochondrial volume per bouton and bouton volume than the animals with a disease score of 2.5. This is consistent with the human form MS, where physical disability and cognitive impairment have also been shown to correlate with gray matter changes (Horakova, Kalincik, Dusankova, & Dolezal, 2012). These groups could however not be statistically compared due to the small number of animals in each group. To verify this hypothesis a larger group size is necessary.

EAE score	Mitochondria/ Bouton	Mitochondrial Volume/Bouton	Synapse Volume	Mitochondria/ area
2.5 (n=2)	2.65	0.46	3.07	0.22
3 (n=4)	2.45	0.27	2.24	0.26
3.5 (n=1)	2.03	0.23	1.71	0.21

Tab. 5-1 Correlation between EAE score and measured parameters in CFP+ mitochondria containing collaterals

Ongoing gray matter pathology even in NAGM in EAE might be a result of the proximal transport blockage and the ensuing dysregulatory mechanisms. This study substantiated previous observations of a net deficit of mitochondria due to a disparity of anterograde and retrograde organelle transport. In addition it demonstrated that even in those axons that appeared healthy (with CFP+ mitochondria) a mismatch in distribution with less mitochondria within the boutons and more in the extra-synaptic parts could be observed. It remains to be discovered whether this is a pathological process or a compensatory one. Nevertheless synapses, even when deprived manage to survive even after weeks of chronic EAE. Future research needs to be directed at finding ways to interrupt this detrimental loss of mitochondria and thereby prevent synaptic pathology and axonal degeneration.

5.4 Back to the roots – healing with vitamins

During the past 10 years a variety of drugs for RRMS have become available. For PPMS and SPMS the story is different. Drugs that have been approved for RRMS have failed trials for PPMS. This lack of therapeutic options might be due to a predominance of neurodegenerative pathology in the second stage of disease. Inflammation is still present, though mainly intrinsic to the CNS behind an intact BBB (Witte et al. 2014, Lassman et al. 2012, Frischer et al. 2009, Magliozzi et al. 2010). Since immunosuppressive treatments do not affect the progressive course long lastingly, one has to direct treatment at limiting axonal degeneration and oxidative stress and inducing remyelination.

Consequently, the current focus of research has shifted from examining processes inside the inflammatory lesion to discovering insidious pathology in the NAWM and NAGM. We know that

the extent of early inflammation correlates with neuronal damage in the progressive phase. However even with the most effective treatments at our disposal the majority of patients with RRMS will develop SPMS after 10 to 20 years (Fitzner & Simons, 2010; Lublin & Reingold, 1996). We are thus in need for therapeutic strategies that target the non-inflammatory components of MS pathology and effectively prevent neurodegeneration.

5.4.1 Targeting mitochondria

As described in detail above, mitochondria have a variety of tasks to fulfill. Their locations are closely regulated and diverse quality control mechanisms collaborate to achieve equilibrium of fission, fusion, recruitment towards the areas of energy demand as well as mitophagy and removal of damaged goods.

From other diseases and physiological conditions we know that in mitochondria under increased oxidative stress, transport no longer functions, ATP production is reduced, Ca^{2+} is released from stores, and fission and fusion processes are altered. These processes lead to disruption of the membrane potential and ultimately to release of cytochrome C and activation of the apoptosis cascade or mitophagy. This depends on the number of mitochondria damaged and the intensity of the toxic stimulus (Witte et al., 2014). Mutations in mitochondrial ATP production, fission and fusion systems, and transport proteins can predispose to a lower capacity to deal with oxidative stress. In Parkinson's disease, mutations in several proteins important for mitochondrial transport or fission and fusion, such as parkin, α-synuclein and PINK1, have been discovered (Federico et al., 2012). In MS, the mitochondrial involvement is not as obvious. While it is quite likely that mitochondrial involvement in MS is of a secondary nature, i.e., caused by inflammation or demyelination it is possible that such acquired mitochondrial dysfunction contributes to progression in the later stages of the disease. In this context it is interesting to note that so far only one mitochondrial mutation has been linked to increased susceptibility for MS. It is found in the promoter sequence of UCP2 known to cause decreased ATP and ROS production (Vogler et al., 2005; Witte et al., 2014). Since mitochondria are a natural source of ROS, which may have important signaling functions, it is not surprising

that an imbalance in regulation can easily upset this tightly regulated system (M. T. Lin & Beal, 2006).

Microglia/Macrophage invasion, myelin breakdown, transport failure, ATP deficiency, channel dysfunction, ion shift and excessive ROS production, all culminate in a metabolic imbalance and energy failure. To break through this chain of events requires a substance that could buffer or even counteract ROS mediated damage. A great part of the intracellular ROS accumulation stems from microglia and macrophages but mitochondria represent a significant source as well, especially when the usual counteractive anti-oxidant pathways fail (van Horssen, Witte, Schreibelt, & de Vries, 2011). Van Horssen suggested non-mutually exclusive theories for increased mitochondrial ROS production. First, upon demyelination mitochondria have been found to accumulate (Hogan et al., 2009). Larger quantities of mitochondrial biomass will ultimately lead to an increased production of ROS. Secondly, mitochondria show structural and functional abnormalities even inside intact axons in inflammatory lesions, which implies that changes in the energy balance of the cell are amongst the first steps to neurodegeneration and could possibly pose a target for therapy (Nikić et al., 2011). Thirdly, mitochondria travel through the entire length of the axon and have a half-life of 30 days. On their way they could accumulate damage to their electron transport chain and ROS production could gradually increase thereby. Fourth, an environment rich in ROS secreted by immune cells will prompt mitochondria to secrete more ROS (van Horssen et al., 2011).

As depicted in Fig. 1-5 excessive mitochondrial ROS production can lead to disrupted signaling cascades, dysfunctional enzymes and a leakage of ROS into the cytosol. Due to this relative lack of anti-oxidant mechanisms it seems reasonable that antioxidative therapy could help slow this detrimental process. In EAE a direct application of a ROS/RNS scavenger could revert mitochondrial changes and transport deficits (Nikić et al., 2011; C. D. Sorbara et al., 2014). Still a local administration in MS is hardly possible. Mao and colleagues used MitoQ a lipophilic cation, triphenylphosphonium, attached to ubiquinone as an intraperitoneally administered agent in treatment of EAE. The MitoQ treated group showed decreased neurological disability and reduced inflammatory markers such as IL-6 (Mao, Manczak, Shirendeb, & Reddy, 2013).

Coenzyme Q10, Vitamin E and resveratrol are also being investigated for their possible use in EAE and clinical MS (Goudarzvand, Javan, Mirnajafi-Zadeh, Mozafari, & Tiraihi, 2010; Nimmagadda et al., 2013; Price et al., 2012; Soleimani, Jameie, Barati, Mehdizadeh, & Kerdari, 2014).

5.4.2 Targeting synapses

Synaptic pathology starts early in EAE. Already at day 7 after induction even before T-cell infiltration and microglial activation an increased turn-over and instability of boutons in the cortex of EAE mice has been reported (Yang, Parkhurst, Hayes, & Gan, 2013). These alterations already begin in the presymptomatic phase of EAE and were found to be mediated by peripheral TNFα production (Yang et al., 2013). TNFα inhibition prevented this change in stability. TNFα inhibition in MS patients though unfortunately led to an increase in attack frequency, duration and severity in a randomized control trial (The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group., 1999). The negative study results are likely related to the pleiotropic nature of TNF that mediates distinct sometimes even opposing effects through its two receptors. For example a positive effect on remyelination of TNFα is mediated by the TNF receptor 2, whereas TNF receptor 1 (TNFR1) signaling contributes to demyelination (Arnett et al., 2001). A more specific approach is warranted for sole treatment of synaptic alterations without influencing oligodendrocyte function and remyelination by selective TNFR1 modification (McCoy & Tansey, 2008).

Several MS drugs are currently being investigated for their specific properties in overcoming synaptic deficits. Electrophysical alterations and dendritic spine loss in EAE for instance can be ameliorated by fingolimod application, while physiological transmission was unaffected (Rossi et al., 2012). Glatirameracetate as well has been shown to protect neurons against glutamate toxicity and synaptopathy in EAE (Gentile et al., 2013). However most of these effects are most likely secondary to immunomodulation.

5.5 Conclusion

In my thesis I was able to demonstrate the distal effects of the transport deficit on mitochondrial distribution in axon collaterals and synapses in chronic EAE.

As Sorbara highlighted in our publication, organelle transport is interrupted in the vast majority of axons in a neuroinflammatory lesion even before structural changes appear (C. D. Sorbara et al., 2014). Whereas short lasting transport interruptions do not affect the distal mitochondrial content, longer lasting transport deficits as observed in models of chronic neuroinflammation decrease the distal mitochondrial content and thereby diminish the energy supply of the distal axonal arbor.

I found boutons depleted of mitochondria and although the number of boutons was not significantly different, the average bouton volume was decreased. It appears that those healthy looking axons that have survived the acute insult, are nevertheless subject to an insidious pathology. One can suspect that this ongoing deprivation ultimately amounts to a deficiency in synaptic function and eventually synaptic and axonal atrophy. This indicates that distal mitochondrial depletion might contribute to gray matter pathology concomitant with clinical deficits seen in EAE and MS. As clinical deficits accumulate over time and are unresponsive to anti-inflammatory treatment in progressive MS, new therapeutic approaches that can restore the axonal homeostasis and supporting mitochondrial function should be explored. Possibly an antioxidative therapy with ROS scavengers could be used as adjuvants to anti-inflammatory therapy.

The picture of multiple sclerosis we see today might not be quite as peculiar anymore as in the times of Carswell and Charcot, still it leaves many questions unanswered. We have a fairly broad choice of disease modifying therapies for RRMS, still a very limited one for the progressive disease stage. With early treatment we can alter the clinical course, but still a large proportion of the patients with RRMS will at some point convert into the secondary progressive stage. Future studies need to be directed at finding substances that can be well tolerated and sustain cell function.

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Eidesstattliche Versicherung

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Ich erkläre hiermit an Eides statt,

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Köln, 03.03.2017

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